Physiological Chemistry and Physics and Medical NMR

Special Issue: Proceedings of the 1st International Symposium Trends in Peptide Research, Perugia, Italy, May 14–18, 1995

Volume 27, Number 4, 1995

PLCHB4 27(4) 231–410 (1995)

Physiological Chemistry and Physics and Medical NMR
Volume 27, Number 4, 1995

Addresses of Chief Editor and Editorial College

Miklós Kellermayer
Department of Clinical Chemistry
University Medical School
Pécs, 7624 Hungary

Janos Ladik
Institute of Physics and Theoretical Chemistry
University of Erlangen-Nurnberg
D-8520 Erlangen
Germany

Søren Løvtrup
Department of Zoophysiology
University of Umea
S-90187, Umea, Sweden

Donald J. L. McIver
Department of Pharmacology
University of Western Ontario
London, N6A, 5C1, Canada

George M. Mrevlishvili
Institute of Physics
Academy of Sciences
380077 Tbilisi
Republic of Georgia

William Negendank
Department of NMR and Medical Spectroscopy
Fox Chase Cancer Center
Philadelphia, Pennsylvania 19111

Toshihiko Ubuka
Department of Biochemistry
Okayama University Medical School
Okayama, 700 Japan

Vasile Vasilescu
Department of Biophysics
Faculty of Medicine
Bucharest 76241, Romania

Denys Wheatley
Department of Pathology
University of Aberdeen
Aberdeen AB9 2ZD, Scotland

EDITORIAL AND BUSINESS OFFICE

Physiological Chemistry and Physics
and Medical NMR
P.O. Box 1452
Melville, New York 11747

Editor In Chief, Dr. Gilbert N. Ling
Managing Editor, Margaret M. Ochsenfeld

SCOPE: PCP provides a forum for the review and publication of reports of original research in a broad range of subjects in biophysics, biochemistry and cellular physiology. Reports of direct applications of basic knowledge to human studies are invited; examples would include the measurements of relaxation times as part of NMR imaging. Single experiments, conclusions based on inadequate statistics, purely speculative papers, and clinical case reports are not accepted.

POLICY: The pages of PCP are accessible to competing viewpoints, interpretations, theories and opinions. Debate is invited via Editorial Comments and Letters to the Editor. Criteria for evaluating submissions include soundness of the study and clarity of presentation, and papers are not rejected on the basis of interpretation or theory, however controversial. PCP believes that scientific issues should be settled by investigation and open debate, not by an appeal to anonymous authority.

PCP attempts to achieve a balance between freedom of expression and constructive peer review. All papers are refereed by reviewers who may remain anonymous, but the Chief Editors make all final decisions, and will handle appeals from authors who feel their papers are unfairly reviewed.

The Editors endeavor to make decisions regarding acceptance or rejection of manuscripts quickly, and have set self-imposed deadlines for doing so. Referees also are given deadlines.

TYPES OF PAPERS: *Regular papers* may be experimental or theoretical. *Short notes, Priority notes* and *Letters* in response to published papers are invited. *Reviews* are desired, but authors are urged to contact an Editor before sending a finished review manuscript. *Symposia* may be published as regular or supplemental issues.

SUBSCRIPTIONS: Price is US$80.00 per volume in the United States and US$87.00 outside the United States. *Physiological Chemistry and Physics and Medical NMR* is published quarterly, 4 issues per year, the volume numbered yearly. New subscriptions will start with the first issue of the volume in progress, unless the subscriber directs otherwise. Most back issues are available

Instructions to Authors

SUBMISSIONS: An original and two copies of all material are requested. The original must be typewritten on one side only, but the copies may be double-sided to reduce cost of mailing. Papers should be sent to Dr. Gilbert N. Ling, Editor, P.O. Box 1452, Melville, New York 11747 U.S.A. Authors, especially those outside of North America, may send their papers to a member of the Editorial College in the same or a nearby country; the member of the college may elect to review the manuscript and/or obtain a second review before forwarding it to the Chief Editors. Manuscripts should be submitted only to this journal and not have been published in part or whole in another publication, except as short preliminary notes, abstracts, or as unpublished work in reviews or symposia. Be sure to keep a copy of your manuscript.

NOTE: Referees will be instructed to destroy their copies of the manuscript after reviewing them. We will return only the original manuscript to you for revision or if rejected.

MANUSCRIPTS: All material should be typed double-spaced with margins at least one inch wide and pages numbered beginning with the title page. *Title Page* should include title (of at most 100 characters and spaces), full names of authors as you wish them to be published, names and cities of institutional affiliation(s) of authors and of institution(s) where the studies were performed, and name, full address and telephone number of the person to whom correspondence, proofs, and reprint requests are to be sent. Three or four *key words* should be listed on the title page.

Abstract should be concise and no longer than 225 words. *Body* may or may not be divided into Introduction, Materials and Methods, Results and Discussion, depending on the length and nature of the paper. Introductory remarks should indicate clearly the significance of the work presented. *References* may be indicated in the text and listed in the reference list in whatever style the author prefers, but we prefer that titles of articles be omitted.

TABLES: Tables should be typewritten on separate sheets and identified by roman numerals (eg. Table III) and titles. Table notes should be keyed by superscript italic lower-case letters (eg. ^{a}Control). The approximate locations of *tables* and *figures* should be indicated in the margins of the manuscript.

EQUATIONS should be broken in a manner estimated to fit into a column 5 inches wide when published. Solidus fraction (a/c) should be used instead of built-up ones ($\frac{a}{c}$) wherever possible.

ILLUSTRATIONS: Original artwork or glossy photostatic prints, together with two photocopies (like Xerox copies) should be provided. Each illustration should be numbered on the back in pencil, along with the authors' names. It is preferred that line drawings be made on paper that is not larger than 8 ½ by 13 inches, and that the drawing be its intended size in the printed paper. Most figures will occupy ½ to full column, that is 5 inches wide. Lines and lettering should be thick enough to allow reduction. A drawing of overall dimension of 8 by 10 inches that will be reduced to ¼ of its original size should be lettered with 18-point (capitals 6 mm high, lower-case 4 mm high) or larger lettering. Graphs and line drawings must be drawn and labeled in black India ink. Glossy photostatic prints are acceptable, and should be attached firmly to a sheet of paper the same size as the manuscript.

PHOTOGRAPHS: High-quality black and white glossy prints should be provided in triplicate, and may be provided as one print plus two photocopies if the photocopies are sufficiently clear to portray the original to referees. Photographs should be attached firmly to a sheet of paper the same size as the manuscript.

REFEREES: Two anonymous referees will be sought for each paper. Authors are encouraged to suggest names and addresses of suitable referees. Referees will be given deadlines for mailing manuscripts back or phoning reviews in, and will be invited to provide editorial statements or Letters that deal with issues raised by submitted papers.

REPRINTS: An order form is enclosed with proofs sent to authors.

PAGE CHARGES: Page charge is $20.00 per published page. It may be waived in the case of severe international exchange difficulties. An additional charge of $5.00 will be levied for photographs that require screening (eg., E.M's, chromatograms, scans).

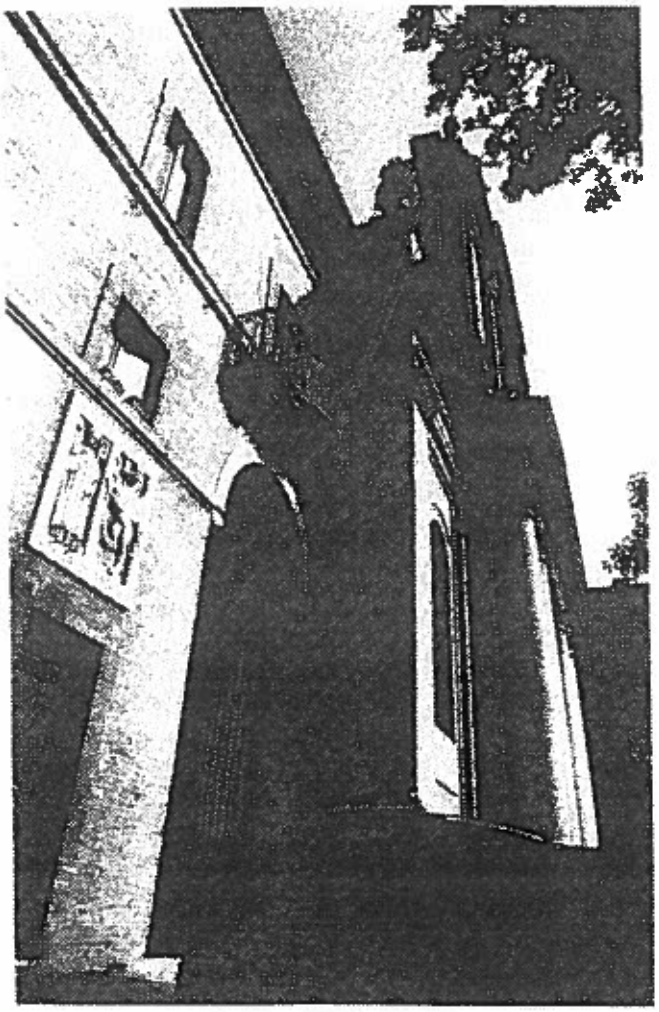
PROCEEDINGS

of the

1ST INTERNATIONAL SYMPOSIUM
TRENDS IN PEPTIDE RESEARCH

Perugia, May 14–18, 1995

organized by
Department of Cellular and Molecular Biology
University of Perugia
Italy



Introduction

DURING THE LAST SEVERAL YEARS progress in understanding, diagnosis and treatment of human desease has been achieved by the awareness that peptides and proteins can regulate essential cellular functions. Some factors have been important in elucidating the role played by proteins and peptides: the discovery of several hormonal peptides and neuropeptides by improved analytical methodology and the ready access to synthetic oligopeptides by synthetic methodology and genetic engineering. For these reasons pharmaceutical scientists routinely use specific peptide sequences as lead structures for drug development. Despite the high level of activity in peptide-based drug research, several serious obstacles hinder the easy development of peptide leads into therapeutically useful agents. Most notable of these obstacles is that of imparting good bioavailability into a peptide-derived drug while maintaining pharmacologic efficacy. Clearly, this problem stems from the unique structural features of peptides, features which are directly linked to their high instability in biological milieu, rapid elimination from plasma and poor transportability across membranes.

The aim of the symposium *Trends in Peptides Research* held at the University of Perugia, was to give scientists having a common interest in studying peptide domains the opportunity to meet colleagues with different backgrounds and experiences. This would allow possible interactions among different disciplines on the topic of peptide research.

The topics discussed during the symposium were:

Determination of peptide structure
Selection of peptides with biological activity
Protein and peptide phosphorylation
Protein-nucleic acid interactions.

Gian Luigi Gianfranceschi
on behalf of the Organizing Committee

2nd International Symposium

TRENDS IN PEPTIDE RESEARCH

University of Calabria
Arcavacata di Rende (Cs), Italy

June 1997

organized by:

Department of Cell Biology
University of Calabria
Arcavacata di Rende (Cs), Italy

Department of Cellular and Molecular Biology
University of Perugia
Perugia, Italy

For more information contact:

Professor G. Martino
Dipartimento di Biologia Cellulare
Università degli Studi della Calabria
87030 Arcavacata di Rende (Cs), Italy
tel.: +39 984 401302
+39 984 839533
fax: +39 984 493160

Professor G.L. Gianfranceschi
Dipartmento di Biologia Cellulare e Molecolare
Sez. di Fisiologia e Biofisica
Università degli Studi di Perugia
06123 Perugia, Italy
tel.: +39 75 5855748
+39 75 5855755
fax: +39 75 5855762

ABSTRACTS

Chemometric Strategies for QSAR and 3D-QSAR Studies of Peptides

Sergio Clementi, Gabriele Cruciani, Manuel Pastor Maeso, Daniela Riganelli, Roberta Valigi, Paola Fifi and Monica Clementi

Laboratorio di Chemiometria, Dipartimento di Chimica, Università di Perugia, Via Elce di Sotto, 10; 06123, Perugia, Italy

Chemometrics and molecular modeling are used as tools for drug design aimed at providing a better understanding of the structural features affecting the biological response. Chemometrics represents the statistical support for Quantitative Structure-Activity Relationships (QSAR) studies; molecular modeling techniques, based on theoretical grounds, allow the study of individual molecules in 3D-space in order to identify sites and types of favorable interactions. In the last few years these two approaches generated a unique 3D-QSAR procedure.

The chemometric methods PCA/PLS seem to be particularly appropriate in QSAR, both for the exploratory analysis of the structural data and for establishing the QSAR under the same descriptor space. The selection of a designed set of informative molecules to best explore the structural variation in the series ensures the reliability of the derived model. Sound predictions of the activity of new molecules are obtained by selecting a few structures by means of design criteria, so that the data base is well balanced, and by using validated models, as in our GOLPE procedure.

In peptide QSAR new structures are usually derived by changing one aminoacid at a time. Sets of peptides obtained in this way do not contain enough information either for ranking the importance of individual aminoacids in affecting biological activity or for providing stable models to be used in predictions. By using statistical design strategies one can select only a few peptides to be synthesized and tested in such a way that the most information is obtained and the most reliable QSAR models derived.

A QSAR table can be prepared describing each aminoacid in a peptide sequence by the statistical descriptors of their side chain called Principal Properties (PP.s), in pairs or triplets: the descriptor matrix. This is the matrix that will be analyzed by PLS in order to find its relationship with the vector measuring the biological activity. PP.s are suitable for use in design criteria to select the molecules needed to form the QSAR table by means of Factorial Designs or D-optimal designs. We used the strategy of Fractional Factorial Designs in PP.s for aminoacids to study a series of peptides behaving as highly selective NK-2 antagonists, and we used D-optimal designs to study the binding of nonapeptides to Major Histocompatibility Complex (MHC) class I proteins, stimulating the interest of immunologists for this chemometric strategy.

A 3D-QSAR is a relationship where the structural descriptors have a 3D nature: these descriptors are usually derived by different modeling techniques. The state of the art in the field easily accepts the CoMFA procedure as the most complete and advanced method for handling the whole problem, while it is also recognized that the GRID method gives accurate estimations of the interaction energies with a wide variety of different probes. Our research group, because of the experience in problem solving by chemometric methods, namely PLS and design, suggested procedures such as GOLPE and ACC to use this new tool at its best.

The drawbacks in 3D-QSAR are strictly linked to the continuity and congruency requirements of such models. The main problem in 3D-QSAR is alignment, which is a consequence of the dependence of the 3D-description upon the position of each molecule within the 3D-grid. We suggested a promising perspective to contribute to the problem of congruency of the 3D-description: the ACC

transforms by means of the PP.s for aminoacids, which allowed us to describe in a congruent way peptides of different lengths. One of the nice aspects of ACC transforms is the interpretation of each element of the ACC vector as the amount of interaction between aminoacids at different lag values, and therefore at different distances. If the description is of this kind, a chemometric model derived thereby might be readable as a map of the receptor, provided the ACC elements describe the level of each interaction for each molecule.

NMR Methods for the Determination of Peptide Structures in Solution

R. Bazzo, D. O. Cicero, G. Barbato, A. Pessi, E. Bianchi and A. Wallace

Istituto di Ricerca di Biologia Molecolare P. Angeletti, via Pontina Km 30.600, 00040 Pomezia, Rome, Italy

Peptide structures in solution are accessible to experimental determination by NMR via the measurement of parameters like NOEs, homo-and hetero-nuclear coupling constants and relaxation times. The degree of definition of "NMR structures" in solution will be discussed and illustrated with the application of classical and new approaches.

Evaluation of Libraries of Compounds

T.J. Mason and L. Dapremont

Chiron Mimotopes Peptide Systems, 10 rue Chevreul, 92150 Suresnes, France

The multipin system allows the parallel synthesis and cleavage of 100's to 1000's of peptides at different scale.

This system applies also to the combinatorial synthesis of peptide libraries. Limitations such as effective representation of each compound in combinatorial libraries will be discussed. The transfer of multipin approach to synthesis of peptidomimetics and small organic molecules will be illustrated by two examples: pin synthesis of N-substituted glycine peptoids, and pin synthesis of dap oligomers.

Log P Determination from 3D-Structure-Water Interactions

Gabriele Cruciani, Paola Fifi and Enrica Filipponi

Laboratorio di Chemiometria, Dipartimento di Chimica, Università di Perugia, Via Elce di Sotto, 10; 06123, Perugia, Italy

Biological membranes play an important role in all living organisms. Drugs may influence the membrane properties increasing the membrane surface, changing the conformation of the anphiphiles molecules or modifying the membrane potential and the hydration of the polar head groups. All these effects may have an important influence on drug activity, therefore the measure of the drug/membrane interaction has become widely used both in the fields of Structure-Activity Relationships and in Structure-Based Drug Design. The Log P parameter is used in order to estimate the membrane affinity for a drug molecule.

The Log P partition coefficient of a molecule is a measure of its relative solubility in the n-octanol and water solvents. Log P had rapidly become an operational definition of hydrophobicity even before the role of hydrophobic bonds in simple organic compounds was clearly understood. Recently, Dunn showed that hydrophobicity depends on solute bulk, polar, and hydrogen-bonding effects and that isotropic surface area (i.e., areas in which no water molecules bind) and hydrated surface areas were nicely correlated with Log P. Tests showed that Log P encodes two major structural contributions, namely a cavity or volume-related term reflecting the energy needed to create a cavity in the solvent, and an interactive term which results from solute-solvent interactions such as dipole-dipole and hydrogen bonds.

Today isotropic and hydrated surface areas and volumes or solute-solvent interactions may be easily computed for all chemical compounds, thus leading towards the computation of parameters highly correlated with hydrophobicity and Log P. These parameters, generated from the three-dimensional structures of the examined compounds, may take into account the conformational effects, the effects of neighboring chemical groups, and also the molecular flexibility of the compounds. Moreover, solute-solvent interactions may be calculated with a dynamic model from a three-dimensional representation of the global solute-solvent system.

The present work proposes a new non-additive and non-fragment based method for calculating Log p values. The methods use the dynamic physicochemical interaction model of a water solvent molecule with the solute molecule in order to evaluate the interaction energies of a water solvent with the hydrophilic and hydrophobic molecular solute regions. The complete 3D representation of the solvent-solute system assures that the chemical groups interactions, charge distributions and the proximity effects are implicitly accounted for by the inclusion of molecular geometry and topology. Finally, an appropriate chemometric procedure (GOLPE) is used in order to correlate the parameters developed by the physicochemical interaction model and the experimental Log P for several hundreds of compounds.

The solution of a compound in a solvent is dependent on the energetics of the interactions between the atoms of the compound and the molecules of the solvent. For a solute molecule of known three-dimensional structure, an interaction field between the solute and the solvent molecules can be calculated. This field describes how the interaction energy varies in the surrounding volume of the solute when a solvent molecule comes in. In the GRID program the interaction field is usually calculated on a regular grid of points superimposed on the solute molecule. To simulate the solvent motion, a water molecule is placed at each grid point and the interaction energy between it and the

solute compound is calculated. The noncovalent interaction energy Exyz is calculated, for each grip point, as the parwise sum of the interaction energies between each atom of the solute compound and each atom of the solvent molecule. The intermolecular energy function consists of the energy due to van der Waals, electronic and H-bonding interactions. Then the grid points of calculated energies can be contoured at a range of energies and displayed using molecular graphics software. The assumption of the method is that lipophilicity, such as other physicochemical properties of compounds, is dependent on their molecular interaction field with water solvent molecules.

Conformational Analysis of β-Turn Mimics Containing a 6-Aminocaproic Acid Linker

David Vander Velde, Osamu Kitagawa, Dinah Dutta, Martha Morton, Fusao Takusagawa, and Heffrey Aubé

Department of Medicinal Chemistry, University of Kansas, Lawrence, Kansas 66045 USA

The β-turn is an important feature of peptide and protein secondary structure. In short linear peptides, β-turns are transient; they can be stabilized by backbone cyclization (particularly in hexapeptides), disulfide linkages, or macrocyclization with nonpeptidic spacers. Such molecules are useful spectroscopic models; small molecules mimicking β-turns have also attracted the attention of medicinal chemists because of the role of β-turns in such events as hormone-receptor and peptide-enzyme recognition. Bioactive molecules such as these might circumvent the well known difficulties in using peptides as orally available drugs.

We have extended the β-turn mimic strategy introduced by Scheraga and coworkers, in which a dipeptide is cyclized with a 6-aminocaproic acid (Aca) linker, to include stereoselective functionalization of the linker unit. Our intent was to use the Aca substituent stereochemistry to affect the conformational preferences of the macrocyclic ring, introducing control over the population of different β-turn types exhibited by the dipeptide. The modified linkers were prepared using stereoselective ring-expansion reactions; simple 1,3 dialkyl substitution (i.e., a 6-amino-3,5-dimethylcaproic acid linker) was initially chosen due to the effectiveness of this pattern in controlling local conformational behavior in acyclic and cyclic molecules. However, it might also be possible to employ the linker as a site of installing other useful functional groups, to impart desirable solubility properties or design in additional macromolecular recognition sites.

NMR and CD data will be presented to demonstrate that changing the stereochemistry of the linker cyclized with Ala-Gly, Gly-Gly, and Ala-Ala dipeptides changes the proportion of type I and type II turn population in solution. X-ray crystallographic data will also be presented, showing both type I and type II turns in the solid state.

Mapping Epitopes on Protein Surfaces

Elisabetta Pizzi and Anna Tramontano

Biocomputing Department, IRBM P. Angeletti, Pomezia (RM), Italy

Both genetically encoded and chemically synthesized peptide libraries have been used for interesting and varied applications in molecular biology. The great diversity contained in a library allows the selection of peptides able to bind to a given receptor, for example an antibody (1).

The library not only provides an enormous number of different amino acid sequences, but each peptide with a given sequence is able to assume a large number of conformations in solution so that, in principle, any structure of the surface of a protein can be mimicked effectively by members of the library.

A protein surface can be viewed as an ensemble of all possible overlapping peptides able to mimic its surface and a peptide library as a collection from which one or more of these peptides can be selected.

If a peptide of the surface ensemble mimics discontinuous regions of the protein, brought together by its tertiary structure, the sequence of the selected peptide will not resemble a region of the linear protein sequence, but rather a patch of exposed residues on its surface which might be impossible to map, even when the tertiary structure of the protein is known. We will describe a method that, given a protein surface, generates a data base containing all the peptides of the "surface ensemble" and that can be used to detect the regions of the protein mimicked by a selected peptide (2).

The method gave very satisfactory results when used to map a discontinuous epitope of a monoclonal antibody raised against human H-ferritin. A data base containing all the peptides of the surface ensemble of H-ferritin was generated and searched with sequences selected from a nonapeptide library displayed on the major coat protein of f1 phage. This allowed us to identify a region of the protein representing the putative discontinuous epitope of the antibody, a result of which is in perfect agreement with all available experimental and theoretical data in the literature about this antigen/antibody complex (3, 4).

References

1. Felici, F., Castagnoli, L., Musacchio, A., Japelli, R. and Cesareni, G. (1991) Selection of antibody ligands from a large library of oligopeptides expressed on a multivalent exposition vector. *J. Mol. Biol.* 222:301-310.
2. Pizzi, E., Cortese, R. and Tramontano, A. (1995) Mapping epitopes on protein surfaces. *Biopolymers 36:*675-680.
3. Luzzago, A., Felici, F., Tramontano, A., Pessi, A. and Cortese, R. (1993) Mapping a discontinuous epitope of human H ferritin using a phage peptide library. *Gene 128:* 51-57.
4. Helmer Citterich, M., Rovida, E., Luzzago, A. and Tramontano, A. (1995) Modelling antibody antigen interactions: ferritin as a case study. *Molecular Immunology 32:*1001-1010.

Mimicry of Molecular Interactions by Phage-Displayed Peptides

Franco Felici, Alessandra Luzzago, Antonella Folgori, Carmela Mennuni, Giuseppe Sgaramella, Claudia Santini, Chantal Santini, Chantal Paolini and Paola Fortugno

Department of Biotechnology, IRBM
(Istituto di Ricerche di Biologia Molecolare P. Angeletti)
via Pontina Km. 30.600, 00040 Pomezia, Rome, Italy

Interaction between molecules is a key step in most biological events. In recent years, the generation of large libraries (composed of 10^7–10^8 variants) of phage-displayed peptides has provided a novel, powerful approach to identifying unknown ligands for a given target molecule.

Libraries have been constructed by cloning oligonucleotides of random sequence in the gene coding for either pIII (Scott and Smith, 1990, *Science, 249:*386–390) or pVIII (Felici *et al.*, 1991, *J. Mol. Biol. 222:*301–310) capsid proteins; in this way, each peptide is displayed on a different phage particle. Phage-displayed peptides thus offer the additional advantage of being physically associated to their genetic information. By using the appropriate target molecule, it is possible to affinity-purify phage displaying a binding peptide sequence from a very heterogeneous mixture of recombinant phage particles. The sequence of the selected peptides can be easily deduced from the sequence of the DNA encapsulated in the same phage particles.

The above selection strategy has been applied to many different ligand/ligate systems, leading to the identification of new peptide sequences which do not necessarily resemble the natural ones, but display analogous binding specificity; even peptides able to mimic non-peptidic molecules have been isolated (Devlin *et al.*, 1990, *Science 249:*404–406; Oldenburg *et al.*, 1992, *Proc. Natl. Acad. Sci. USA 89:*5393–5397; Hoess *et al.*, 1993, *Gene 128:*43–49).

By using monoclonal antibodies (mAbs) for the selection, peptide mimics (*mimotopes*) of the original epitope have been selected. The molecular basis of mimicry is that an antibody is the negative image of the antigen, and that a peptide specifically binding to the antigen-binding site of the antibody might be a positive image of the antigen.

The selection of peptide mimics has been achieved not only for mAbs directed against linear proteic epitopes, but also for antibodies recognizing discontinuous ones (where residues constituting the epitope are close in space in the folded protein but distant from one another in the primary sequence). In the former case, a "core" of residues conserved among families of selected peptides showing significant similarity to specific portions of the natural antigen is usually identified (Cortese *et al.*, 1994, *Trends in Biotechnol. 12:*262–267). Discontinuous epitopes are a different case, and have been mapped only when a detailed three-dimensional structure of the antigen was available (Luzzago *et al.*, 1993, *Gene 128:*51–57; Felici *et al.*, 1993, *Gene 128:*21–27). If the mimicry is sufficiently accurate the selected peptides could be suitable substitute immunogens, completely distinct from the original antigen (Folgori *et al.*, 1994, *EMBO J. 13:*2236–2243; Orlandi *et al.*, 1994, *Europ. J. Immunol. 24:*2868–2873).

Inhibitors of IL-6/IL-6-Receptor Binding Selected from a Multimeric Synthetic Combinatorial Peptide Library

A. Wallace[1], S. Altamura[2], C. Toniatti[2], E. Bianchi[1], P. Delmastro[3], G. Ciliberto[2] and A. Pessi[1]

Departments of [1]Biochemistry, [3]Immunology and [2]Genetics, Istituto di Ricerche di Biologia Molecolare P. Angeletti (IRBM), Via Pontina Km 30.600, 00040 Pomezia (Rome), Italy

We describe here a novel type of SCPL, named Multimeric Synthetic Peptide Combinatorial Library (M-SPCL), in which the degree of multiplicity of the randomized peptide sequences can be varied in a controlled way (Wallace *et al.*, 1994). The library, which was prepared by solid-phase peptide synthesis as described (Houghten *et al.*, 1991; Philla *et al.*, 1992), is based on the structure of Multiple Antigen Peptides (MAP, Tam, 1988). While, due to its synthetic nature, M-SPCL retains the advantage of the possible inclusion of D- and uncoded amino acids, as well as non-peptide moieties in the library, the high density assembly of the peptide sequences on the branching scaffold is analogous to the presentation of multiple copies of the epitope in phage libraries (Scott & Smith, 1990). Likewise, selection from these libraries is most probably avidity-driven, thereby effectively lowering the binding threshold for the selection of ligands. Moreover, in a number of cases, multimericity of the peptide sequence may be a desirable feature *per se*: first, since multimeric peptides provide increased sensitivity and reliability compared to linear peptides in solid-phase immunoassays (Tam & Zavala, 1989), such libraries could be directly screened to find immunoassay reagents; second, some hormones bind to dimeric forms of their receptor, dimeric peptides have been shown to alter the assembly of essential viral proteins, and DNA binding of regulatory proteins often takes place in the form of dimers; third, multimerization may substantially increase the binding affinity (Fassina *et al.*, 1992 a, b). The usefulness of this M-SPCL was demonstrated by the selection of octameric peptides which specifically inhibit the binding of the cytokine human Interleukin-6 (hIL6) to a soluble form of its receptor (hIL6R), with an apparent affinity in the sub-micromolar range. Tetrameric, but no dimeric, branched peptides based on the same sequences are also active with comparable affinity. Neither this sequence, nor any other sequence active in this system, could be selected from the corresponding linear pentapeptide SPCL.

References

Fassina, G., Corti, A. and Cassani, G. (1992a) *Int. J. Peptide Protein Res. 39:*549-556.

Fassina, G., Cassani, G. and Corti, A. (1992b) *Arch. Biochem. Biophys. 296:*137-143.

Houghten, R.A., Pinilla, C., Blondelle, S.E., Appel, J.R., Dooley, C.T. and Cuervo, J.H. (1991) *Nature 354:*84-86.

Pinilla, C., Appel, J.R., Blanc, P. and Houghten, R.A. (1992) *BioTechniqes 13:*901-905.

Scott, J.K. and Smith, G.P. (1990) *Science 249:*386-390.

Tam, J.(1988) *Proc. Natl. Acad. Sci. USA 85:*5409-5413.

Tam, J.P. and Zavala, F. (1989) *J. Immunol. Meth. 124:*53-61.

Wallace, A., Altamura, S., Toniatti, C., Vitelli, A., Bianchi, E., Delmastro, P., Ciliberto, G. and Pessi, A. (1994) *Peptide Res. 7:*27-31.

Antimicrobial Peptides from Amphibian Skin

Maurizio Simmaco[1], Guiseppina Mignogna, Silvia Canofeni and Donatella Barra

Dipartimento di Scienze Biochimiche, Università di Roma La Sapienza and CNR, Centro di Biologia Molecolare. [1]Istituto di Scienze Biochimiche, Università di Chieti, Italy

Dermal glands of amphibians represent a rich source of biologically active compounds such as pharmacologically active and antimicrobial or hemolytic peptides. These molecules represent an additional immune mechanism of host defense, similarly to other antimicrobial peptide systems found in different cellular types of higher eukaryotes. The antimicrobial peptides from amphibian skin can be stored in cutaneous granular glands, after processing of their precursors, as active components easily available. These molecules are cationic but otherwise differ considerably in such basic features as their size, presence of disulfide bonds, and structural motifs. The fact that many antimicrobial peptides are able to adopt amphipathic α-helix structures in hydrophobic environments suggests formation of ion-channel pores in the target membrane and indicates that cell death is a consequence of an altered membrane permeability. This hypothesis is confirmed by the results of experiments performed with synthetic peptides containing all D-amino acids. Moreover, the presence of positively charged residues in these peptides explains their selectivity, in that some of them interact with the anionic pospholipids present in the bacterial membrane but not with erythrocyte membranes which contain little quantities of acidic phospholipids.

We have investigated in detail the peptide fraction possessing antibacterial activity from skin secretions of two European frogs, *Bombina variegata* and *Rana esculenta*. In both species, several peptides families were found. The diversity among the various families and the presence of more than one molecular form within each family seem to provide a basis for a wide range of antimicrobial activity. From *Bombina variegata* a family of hydrophobic peptides possessing both antibacterial and hemolytic actives was found, which presents a striking feature in that they contain in their sequence a D-alloisoleucine residue. The occurrence of this residue is not essential for cytolytic activity, but probably contributes to the biostability of the peptide. In *Rana esculenta*, several antimicrobial peptides of different length and distinctive activity were described, which invariably contain a common structural motif, i.e., an intramolecular disulfide bridge located at the carboxyl-terminal end forming a seven-member ring. A very high hemolytic activity is displayed by a 23-residue peptide from *Rana esculenta* and the most potent antimicrobial activity is due to a 46-residue peptide from the same species.

Structural Features and Biological Activities of Novel Mammalian Antibiotic Peptides Identified Through Amplification of Similar cDNA Sequences

Magherita Zanetti[1,2], Marco Scocchi[1], Barbara Skerlavaj[2], Paola Storici[3], Alessandro Tossi[3], Luigi Bagella[1], Angela Risso[2], Domenico Romeo[3] and Renato Gennaro[2]

[1]Laboratorio Nazionale Consorzio Interuniversitario Biotecnologie, AREA Science Park, Padriciano, 1-34012 Trieste, [2]Dipartimento di Scienze e Tecnologie Biomediche, Università di Udine, 1-33100 Udine, [3]Dipartimento di Biochimica, Biofisica e Chimica delle Macromolecole, Università di Trieste, 1-34127 Trieste, Italy

Over the past several years a variety of peptides and polypeptides that exert direct and selective bactericidal effects *in vitro* have been isolated from several organisms. Unlike most of the "classical" antibiotics, which are made through a complex enzymatic synthesis, these peptides are made from gene-encoded precursor proteins and are utilized by animals and plants to kill microorganisms in different physiological settings. In arthropodes, they are secreted into internal body fluids (hemolymph). In vertebrates, they protect the mucosal epithelia of amphibia and mammals, and provide an important component of the non-oxidative microbicidal activity of mammalian phagocytes. Although displaying a marked inter-species diversity in structure (more than 100 different antimicrobial peptides have so far been identified), they share some common features, such as a high content of basic residues and the tendency to adopt an amphipathic conformation which is mandatory for their functioning as membrane-active molecules.

We have recently recognized the existence of a group of precursors of antimicrobial peptides from bovine neutrophils, in which a highly variable C-terminal region showing antimicrobial activity after processing, is preceded by an N-terminal, highly conserved preproregion. We have used a molecular biological approach based on the high conservation of the 5′ region of the corresponding mRNAs (RACE protocol based on 5′ and 3′ end amplification of similar cDNAs) to amplify myeloid bovine and porcine cDNAs encoding novel antimicrobial peptides. Several different polypeptides were deduced from the amplified cDNAs. All showed very similar preproregions and structurally varied and highly cationic C-terminal regions. The sequences, and secondary structure prediction analysis of the C-terminal regions indicated that these may assume an amphipathic conformation. Peptides corresponding to five of these sequences were chemically synthesized and termed PMAP-23, PMAP-36, PMAP-37 (from Pig Myeloid Antimicrobial Peptides of 23, 36 and 37 residues), BMAP-27 and BMAP-28 (from Bovine Myeloid Antimicrobial Peptides of 27 and 28 residues). Their biological activity, structure and mechanism of action have been characterized. CD spectroscopy shows that PMAP-36, PMAP-37, BMAP-27 and BMAP-28 undergo a transition from a random coil to an ordered, mainly helical structure on addition of trifluoroethanol. The spectrum of PMAP-23 is not incompatible with a tentatively assigned β hairpin-type structure. All these peptides exert a potent antimicrobial activity against various Gram-negative and Gram-positive bacteria at μM concentrations. Some of them show antifungal activity against *Cryptococcus neoformans* and *Candida albicans*. The antibacterial activity appears to be correlated to their ability to perturb the membranes of target cells. All of them cause a rapid permeabilization of the inner membrane of *E. coli*, which is induced at concentrations comparable to the antibacterial values. Some also display hemolytic activity, and are cytotoxic against several human tumor cell lines.

This molecular biological approach, combined with structure prediction and chemical synthesis, has allowed us to rapidly identify novel antimicrobial peptides potentially useful as human therapeutic agents or as model compounds for the design of novel antibiotics.

Role of a Lymphocytic Low-Molecular Weight Peptide in Acquired Immunodeficiency

V.E.M. Rosso di San Secondo, A. Aniasi, [1]G. Lugaro, P.C. Montecucchi and G. Sirchia

Transplantation Immunology, University Hospital; [1]Istituto di Chimica degli Ormoni, CNR, Milano, Italy

A hydrophilic low-molecular weight peptide (about 1 kDa) that we have purified from peripheral blood mononuclear cells and named lymphocytic suppressor factor (LSF) is able to suppress antigen- and mitogen-induced lymphocyte transformation and prolong allograft survival times in C57b/6N mice transplanted with skin from C3H/HeN mice. At the molecular level LSF acts by inhibiting replicational and transcriptional processes in intact cells, isolated nuclei and cell-free systems. Combined with cyclosporine-A (CsA) LSF showed an additive effect on *in vitro* CsA-induced inhibition of mitogen-induced lymphocyte proliferation. Moreover, intracellular content of LSF increased with the *in vivo* increase of CsA-induced immunosuppression. These data strongly suggest that LSF participates as intracellular regulator of replicational and transcriptional processes in the development and persistence of acquired immunodeficiencies.

Modification of Secreted Protein by Cultured Cells in the Culture Media

G. Rebel - UPR 9003 Du CNRS

IRCAD-Hopitaux Universitaires-BP426-F67091 Strasbourg Cedex, France

Protein stability is an important consideration when recovering proteins secreted in culture media by cells. Indeed, while secreting proteins and peptides with biological properties such as trophic factors, growth factors, etc., cultured cells also secrete various proteases in the culture media which may strongly jeopardize further studies to be performed on the protein of interest. Usually, cells themselves are protected against media proteases by nexins, while secreted proteins are not. In order to avoid the alteration or even the complete degradation of secreted proteins, care must be taken to counter proteases effect. Though, less well known other modifications result from the interaction of the secreted proteins with some media components such as lipids, glucose etc.. Using various examples, the importance of this protein alteration will be analyzed.

Molecular Models of Peptides by Mass Spectrometry Analysis

G. Lugaro[1], L. De Angelis[2], F. Giavarini[3], V. Marsili, L. Manicinelli, E. Castigli and G.L. Gianfranceschi

[1]Istituto di Chimica degli Ormoni, C.N.R., Milano, Italy; [2]Istituto di Scienze Farmacologiche Università di Milano Dipartimento di Biologia Cellulare e Molecolare, Università di Perugia, Italy

We have previously reported the characterization of low molecular weight peptides involved in the control of RNA synthesis in cell and cell-free systems (1, 2). They were isolated from biological fluids such as blood and seminal plasma or from the chromatin of several tissues. The peptide fraction purified from different sources appears composed of strongly related compounds with similar amino acid composition and structure. Despite the difficulty represented by the presence of a family of similar peptides and by the small amount of available purified peptide some biochemical features have been demonstrated: i) a molecular weight of about 1000; ii) the blocked N-terminal probably constituted by pyroglutamic acid; iii) the predominant presence of glutamic acid (3-6 residues), aspartic acid (1-3), serine (1), glycine (1) and alanine (1); iv) a phosphoric group bound to serine.

In this work we report the design of molecular models performed on the basis of combined information obtained from biochemical and mass spectrometry analysis of native peptides isolated from bull seminal plasma or pea buds and trout testis chromatin. The mass spectrometry analysis gives useful information on the possible structure, also in a mixture of peptide sequences, because we obtain the exact molecular weights of the native molecules together with the molecular weights of their possible breakdown products. Some of the peptide molecular models have been synthesized. The biological activity of the native and synthetic peptides has been studied on the following systems: a) DNA transcription in reconstituted systems *in vitro* (3); b) RNA synthesis in isolated nuclei from rat hepatocytes or PC12 cells (4); c) growth and differentiation of HL60 human leukemia cells (5).

References

1. Lugaro, G., Campagnari, F., Moretti, R. and Casellato, M.M. (1988) *Biochim. Biophys. Acta 950:*420-428.
2. Chillemi, F., Lugaro, G., Boari, D., Cardellini, E., Bramucci, M., Miano, A., Amici, D., Gianfranceschi, G.L. and Durban, E. (1991) *FEBS Lett 291:*67-70.
3. Gianfranceschi, G.L., Czerwinski, A., Angiolillo, A., Marsili, V., Castigli, E., Mancinelli, L., Miano, A., Bramucci, M. and Amici, D. (1994) *Peptides 15:*7-13.
4. Castigli, E., Mancinelli, L., Mariggiò, M.A. and Gianfranceschi, G.L. (1993) *Am. J. Physiol. 265(Cell Physiol. 34):*C1220-C1223.
5. Marsili, V., Angiolillo, A. and Gianfranceschi, G.L. (1995) *Cell. Mol. Biol.*, in press.

Low Molecular Weight Peptide from Calf Liver Mitochondrial DNA: Structure and Aminoacidic Sequence

Angela Spena, Rosa Chimenti, Concetta Covello, Tiziana De Cicco, Sergio Mazzulla and Guglielmo Martino

Department of Cell Biology University of Calabira, 87030 Rende (CS), Italy

Authors isolated a peptide fraction from mitochondrial DNA of calf liver by the method of Drouin (Drouin, J., 1980, *Med. Biol. 140:*15). This peptide fraction, released at pH 9.5 from extensively purified mitochondrial DNA (Coderoni, S. *et al.*, 1988, *Physiol. Chem. Phys. Med. NMR 20:*91), exerts *in vitro* a regulatory role on the transcription and duplication activity of DNA (Spena A. *et al.*, 1993, *J. Biol. Res., LXIX:*755). The structural analysis was performed on both crude extract and on fractioned peptides. Spectroscopical analysis was performed on the total extract. U.V. visible data were obtained in water with a Shimadzu U.V.-2100 spectrophotometer. I.R. spectra were performed using a Perkin-Elmer 1330 infra-red spectrometer on KBr pellets. ^{31}P NMR spectra were performed using a Bruker 300 MHz spectrometer; spectra were related to orthophosphoric acid at pH 6.7. The peptide fraction was also submitted to fractionation procedures by the fingerprinting techniques of Hitz *et al.* (Hitz, H. *et al.*, 1977, *Eur. J. Biochem. 77:*497; Heiland, I. *et al.*, 1976, *Hoppe-Seyler's Z. Physicol. Chem. 357:*1751; Chen, R., 1977, *Hoppe-Seyler's Z. Physicol. Chem. 358:*1415). This peptide fraction is composed of two sub-fractions with different electrophoretic mobility in 2% pyridine, 4% acetic acid, 15% acetone, 79% water, at 10 mA, 250 V, on a 20 × 20 sheet Polygram Cel 300. The fingerprint of the mitochondrial peptide fraction has four sub-fractions only by ascendent chromatography in 7.5% acetic acid, 25% pyridine, 37.5% n-buthanol, 30% water. The four mitochondrial sub-fractions are red colored after reaction with ninhydrin. Among the four isolated sub-fractions only the one slowest in chromatography and fastest in electrophoresis has been sequenced; here we present its aminoacidic sequence determined by the manual microsequence of Chang (Chang, J.Y. *et al.*, 1978, *FEBS Lett. 39:*205). The amino acids sequence of the main chain is mainly composed of the Threo amino acid, which is most likely modified.

Biologically Active Pyroglutamyl N-Terminal Oligopeptides: Parts of Larger Molecules?

Kjell Elgjo[1], Karl L. Reichelt[2] and Wenche H. Reichelt[2]

[1]Institute of Pathology and [2]Pediatric Research Institute,
The National Hospital (Rikshospitalet), N-0027 Oslo, Norway

In 1984, we identified and characterized a growth-inhibiting pentapeptide, pyroGlu-Glu-Asp-Ser-GlyOH, from mouse epidermis. Later, other pyro-Glu N-terminal oligopeptides have been isolated and characterized from liver and mouse intestine. Experiments, mainly done with the epidermal pentapeptide, have shown the following:

- Effect on cells in different phases of the cell cycle (late G_1, S, and G_2)
- Reversible inhibitory effect? (*In vitro* experiments indicate an effect on terminal differentiation)
- β-receptor blockage abrogates the inhibitory effect on cells in G_2, but not on cells in late G_1/S
- Effect on malignant cells belonging to the same type of differentiation (NB: only a limited number of experiments)
- Refractory period after a treatment
- Repeated treatments over a period of several days have little effect.

In order to find how and where the pyroGlu N-terminal growth-modulating peptides find their proper place in the current map of intercellular communication, we are now engaged in the following experiments:

- Are the peptides part of larger molecules?
- How are they related to other growth-modulating factors?
- Are they coded for by genes that are related to known growth regulating proto-oncogens, especially to growth suppressing genes?
- Can antibodies against the characterized peptides be used in clinical medicine, e.g., detection of peptides in the urine, serum/plasma in patients with cancer in the related organs?

Currently, we are using affinity columns coated with pyroGlu-Glu-Asp-Ser-GlyOH to find molecules in epidermis homogenates that bind to the peptide, and western blotting techniques to identify molecules in epidermis homogenates that bind to a polyclonal rabbit antibody against pyroGlu-Glu-Asp-Ser-GlyOH. These experiments could help us identify soluble parts of a receptor, or a carrier protein, as well as finding a larger molecule of which the pentapeptide is a part. Preliminary results of these experiments will be presented and discussed.

Purification and Characterization of Swine Serum Enzyme Which Hydrolyzes Epidermal Inhibitory Pentapeptide

M. Bramucci, A. Miano, L. Quassinti, E. Maccari, O. Murri and D. Amici

Dipartimento di Biologia Molecolare, Cellulare e Animale, Università di Camerino, Via F. Camerini n2, I-62032 Camerino (MC), Italy

Introduction: Several low molecular weight growth-inhibiting peptides have been isolated from different tissues. These peptides act preferentially on the tissues in which they are produced, even though they are structurally very similar,. We were interested in one such growth inhibitory pentapeptide, pyroGlu-Glu-Asp-Ser-Gly (EPP), which has been isolated from skin and epidermal extracts (1). This epidermal pentapeptide inhibits both mithosis and DNA synthesis in mouse epithelium *in vivo* and in keratinocytes cell lines *in vitro*. It enhances differentiation in primary cultures of mouse epidermal cells and in transformed mouse epidermal cells *in vitro*. Little is known of the mode of action, degradation, or metabolism of EPP, moreover it is observed to be unstable in culture medium. Whitehead *et al.* (2) have demonstrated the presence in mammalian, avian and reptilian sera of enzyme which rapidly cleaves EPP. In this report we describe the purification to molecular homogeneity of the enzyme from swine serum which cleaves EPP.

Materials and Methods: Swine serum was precipitated by fractionation between 35-65% saturation of $(NH_4)_2SO_4$. The active fraction was dialyzed and chromatographed on a DEAE-cellulose (DE52, Whatman), Phenyl-Sepharose (Sigma), Sephacryl S200 (Pharmacia) and Resource Q (Pharmacia) columns. The active fraction was analyzed by SDS/2-mercaptoethanol polyacrylamide gel electrophoresis. The EPP hydrolytic activity was measured following the incubation of synthetic EPP in 50 mM Tris/HCl buffer, pH 7.4, containing 150 mM NaCl at 37ºC with aliquots from different steps of purification. The resulting products were separated by reverse phase HPLC analysis using Supelcosil LC318 (Supelco) column.

Results: We have carried out the purification from swine serum of the enzyme responsible for the EPP hydrolysis. The active fraction from the last step of purification was analyzed by SDS/PAGE showing one single band which migrates as a protein with an apparent molecular weight of 180,000 Da. The periodic acid-Schiff stain of the band in SDS/PAGE was positive proving the presence of carbohydrates bound to the enzyme. EDTA, EGTA, or *o*-phenanthroline in the millimolar concentration range significantly inhibited the activity, suggesting a metalloendopeptidase character of the enzyme. The Zn^{2+} ion was able to restore the activity after the treatment of the enzyme with *o*-phenanthroline. The amino acid analysis of EPP metabolites and other synthetic peptides proved the enzyme was a dipeptidyl carboxypeptidase. Dipeptidyl carboxypeptidase specific inhibitors are used to characterize more the serum enzyme. Captopril and Lisinopril quite inhibited enzyme activity at micromolar concentration, suggesting the presence of angiotensin-converting enzyme-like activity. The hydrolysis of angiotensin I by swine serum enzyme and the hydrolysis of EPP by lung rabbit angiotensin-converting enzyme confirm the presence of dipeptidyl carboxypeptidase enzyme activity like-ACE responsable of hydrolysis of EPP.

References

1. Reichelt, K.L., *et al.* (1987) *Biochem. Biophys. Res. Commun. 146:*1493-1501.
2. Whitehead, P.A. *et al.* (1991) *Biochem. Biophys. Res. Commun. 175:*978-985.

Selection of Peptides with Pre-Determined Structure from a Conformationally Homogeneous Combinatorial Peptide Library

Elisabetta Bianchi, Antonella Folgori, Andrew Wallace, Maria Nicotra, Stefano Acali, Armelle Phalipon[1], Gaetano Barbato, Renzo Bazzo, Riccardo Cortese, Franco Felici and Antonello Pessi

IRBM P. Angeletti, Via Pontina Km 30.600, 00040 Pomezia (Rome), Italy and [1]Institut Pasteur, Paris, France

In order to realize the full potential of combinatorial methods for drug discovery the emphasis and the present sophistication in the design and synthesis of individual molecules must be shifted to the design of populations of (selectable) molecules (Gordon *et al.*, 1994). In the case of peptides, much effort is being invested in the search for a rational way to convert the information encoded in peptide ligands into peptidomimetics. We believe that major progress in this direction would come from the availability of peptide libraries of predetermined conformation. By coupling the power of selection methods with the rational design of conformation-inducing templates for the selectable sequences one could envisage a process which we term selection-driven peptidomimetic design, whereby a conformational model for the peptide pharmacophore is directly derived from the screening, prompting the design of a suitable non-peptidic scaffold to replace the peptide backbone. We describe here the first example of a conformationally homogeneous combinatorial peptide library, based on the α-helical geometry, which yields ligands with the expected pharmacophoric structure upon selection (Bianchi *et al.*, 1995). The library was built by randomizing five positions in the α-helical portion of a 26 amino acid Cys_2His_2 consensus 'zinc-finger' motif. Since in zinc fingers metal coordination and folding are coupled, in our library metal-dependent binding represents a built-in control against the selection of structurally undefined sequences. The α-helical library was produced both as fusion with the pVIII protein of filamentous phage and as soluble peptides by chemical synthesis, the latter enabling the expansion of the selectable repertoire by the inclusion of non-coded amino acids. The two libraries were independently screened with the same receptor (a monoclonal IgA reactive against the LPS of the human pathogen *Shigella flexneri*) yielding a very similar consensus. In particular, the peptides defined by both methods showed a very strong, zinc-dependent binding to the IgA. The geometrical arrangement of the side chains of the selected peptide pharmacophore was shown by Circular Dichroism, Co(II)-complex absorption and high-resolution NMR to be structurally invariant with respect to the parent zinc-finger. The design of a template for the corresponding scaffolded peptidomimetic will also be described.

References

Gordon, G., Barrett, R.W., Dower, W.J., Fodor, PA. and Gallop, M.A. (1994) *J. Med. Chem. 37:*1385-1401.

Bianchi, E., Folgori, A., Wallace, A., Nicotra, M., Acali, S., Phalipon, A., Barbato, G., Bazzo, R., Cortese, R., Felici, F. and Pessi, A. (1995) *J. Mol. Biol. 247:*154-160.

Rational Design of NK-1 Substance P Receptor Antagonists Based on Aminocycloalkyl Residues

A. Sisto, F. Centini, C.I. Fincham, P. Lombardi, E. Monteagudo, E. Potier and R. Terracciano

Dipartimento di Chimica, Menarini Ricerche Sud, Via Tito Speri 10, 00040 Pomezia (RM), Italy

The investigation of both the structural and dynamic features that regulate the recognition of the target receptor is a requisite for the rational modification of a biologically active compound. In a program aimed at the synthesis and biological evaluation of novel, potent NK-1 tachykinin receptor antagonists, a hypothesis of pharmacophoric group topology was elaborated from the comparison of the low energy structures for a series of peptide (FK-888) and non peptide (SR 140333, CP 96345, CP 99994, CGP 47899) Substance P antagonists, and was used to plan further modification of the molecule. The comparison was performed by superimposing the aromatic rings (whose presence is crucial for the biological activity), assuming that the rest of the molecule behaved predominantly as a template to arrange the key aromatic groups in the right position in space. A series of 2-aminocyclohexanecarboxylic acid analogues were selected to replace the Hyp in the Fujisawa antagonist FK 888, as the best templates for reproducing the postulated topology of the aromatic rings.

The application of spectroscopy techniques to the resulting analogues, all showing an interesting biological activity in comparison to FK 888, revealed a linear correlation between the stacking propensity of two aromatic rings and the binding affinity.

By utilizing our model of the bioactive structure in further molecular modeling studies, we identified the 1-aminocycloalkylcarboxylic acid residues as more productive templates among the family of cycloalkyl amino acids. The resulting analogues showed, in conformational studies via the simulated annealing procedure, a high structural rigidity and a close reproduction of FK 888's aromatic ring assembly. The most promising candidate was synthesized (MEN 10930), and showed an inhibition of Substance P binding to NK-1 receptor in the nanomolar range.

Acellular Pertussis Vaccine Composed of Genetically Inactivated Pertussis Toxin

Samuele Peppoloni, Mariagrazia Pizza, Teresa De Magistris, Antonella Bartoloni and Rino Rappuoli

IRIS Research Institute, BIOCINE Spa, Via Fiorentina 1, 53100 Siena, Italy

Whooping cough, an acute respiratory disease affecting over 60 million infants, can be prevented by vaccination. The vaccine currently used, composed of killed bacterial cells, however, has been associated with many side effects. An improved vaccine against the disease should contain pertussis toxin (PT), a major virulent factor of *Bordetella pertussis*. In order to be included in the vaccine, PT needs to be detoxified and the chemical methods used so far are not completely satisfactory, since they give a product with reduced immunogenicity and possible residual toxicity. To avoid this problem, we have used recombinant DNA technologies to clone the PT gene, express it in bacteria, map the B and T cell epitopes of the molecule and identify the amino acids that are important for the enzymatic activity and toxicity. Based on this information, the gene coding for PT was mutated to produce an inactive protein. This genetically modified PT is non toxic, highly immunogenic and able to protect mice from intracerebral challenge with virulent *Bordetella pertussis*. The mutant was included as a main component of an acellular pertussis vaccine which has been shown in numerous clinical trials to be more safe and immunogenic than the old cellular vaccine.

Molecular Analysis of the Functional Domains of Protein Kinase-CK2 (casein kinase-2)

Stefania Sarno, Brigitte Boldyreff[1], Flavio Meggio, Philippe Vaglio, Olaf-G. Issinger[1] and Lorenzo A. Pinna

Dipartimento di Chimica Biologica, Università di Padova, Padova, Italy and [1]Institut für Humangenetik, Universität des Saarlandes, Homburg, Germany

Protein kinase CK2 is a ubiquitous Ser/Thr specific protein kinase responsible for the phosphorylation of a myriad of protein substrates (> 100 identified to date) which are implicated in a variety of cellular functions, with special reference to signal transduction and gene expression. The acronym CK2 is derived from the artificial substrate, casein, but the full name (casein kinase-2) should be abandoned since CK2 is quite distinct from the genuine casein kinase(s) responsible for the *in vivo* phosphorylation of caseins. CK2 holoenzyme almost invariably consists of very stable heterotertamers composed of two catalytic (α and/or α′) and two non catalytic, β-subunits. Under basal conditions CK2 is spontaneously active toward many of its substrates; some substrates however require the presence of very basic (poly)peptides in order to be appreciably phosphorylated. CK2 basal activity actually results from the balance between positive and negative regulations imposed by the β-subunit. Reconstitution experiments with variably deleted or mutated β-subunits and/or with synthetic β-subunit domains revealed that while the "positive" functions (increased stability and enhanced catalytic activity toward canonical substrates) reside in its C-terminal domain, an acidic region in the N terminal domain is responsible for intrinsic downregulation which prevails with those substrates, like calmodulin, which are almost unaffected under basal conditions. The same N terminal region also confers susceptibility to polybasic peptides, which appear to stimulate activity by interacting with the acidic residues otherwise responsible for negative regulation.

Although the β-subunits deeply influence the target selectivity of CK2, the site specificity, determined by multiple acidic residues surrounding the target aminoacid (mostly down-stream from it) depends on structural features of the catalytic subunit. By site directed mutagenesis of the α-subunit a number of basic residues implicated in site recognition, inhibition by polyanionic effectors and negative regulation by the β-subunit have been identified. In particular H^{160} contributes to the recognition of an acidic residue at position -2 relative to the target aminoacid; R^{191},K^{195},R^{198} critically and specifically recognize the acidic determinant at position +1 whereas two adjacent basic clusters ($K^{74}KKK^{77}$ and K^{79},R^{80},R^{83}) variably interact with the acidic determinants between positions +2 and +5 (and possibly also farther). The K^{74}-K^{77} cluster, but not the other basic residues which have been mutated, is also responsible for inhibition by heparin, while inhibition by the pseudosubstrate EEEEEYEEEEEEE is compromised by mutations in any of the three basic clusters. The same basic clusters plus another basic triplet (278-280) are variably implicated in downregulation by the β-subunit N terminal domain.

Altogether these data disclose a situation where a number of structural elements are utilized for multiple functions thus contributing to a subtle and integrated modulation of CK2 activity/targeting.

Phosphorylation by Protein Kinase CKII of Acidic Peptides and Proteins Domains

Elena Cardellini, Antonella Angiolillo and Gian Luigi Gianfranceschi

Dipartimento di Biologia Cellulare e Molecolare, Università di Perugia, Perugia, Italy

Low molecular weight phosphorylated peptides, involved in RNA transcription, have been isolated from the chromatin of several tissues (1).

Amino acid analysis of isolated peptides, showing a high amount of glutamic acid, aspartic acid and serine, suggested these peptides as possible substrates for protein kinase CKII (pCKII).

Phosphorylation by CKII has been demonstrated to modulate activity of several proteins (transcription factors, DNA topoisomerases, RNA polymerases, oncogenes and suppressor genes) (2).

Acidic peptides, synthesized on the basis of biochemical and mass spectrometry analysis obtained from native peptides, were examined to study substrate requirements for pCKII (3, 4).

Because nuclear proteins, such as topoisomerase I and RNA polymerase II, have been found to contain sequence similar to that of native peptides, these domains were synthesized and demonstrated to be good substrates for pCKII (5, 6). A possible involvement of these phosphorylated domains in interactions between proteins and DNA was supported by the finding that proteins tightly bound to DNA are efficiently phosphorylated by pCKII.

References

1. Gianfranceschi, G.L., Czerwinski, A., Angiolillo, A. *et al.* (1994) *Peptides 15*:7-13.
2. Pinna, L.A. (1990) *Biochim. Biophys. Acta 1054*:267-284.
3. Gianfranceschi, G.L., Cardellini, E. and Piccinini, G. *et al.* (1989) *Physiol. Chem. Phys. & Med. NMR 21*:289-294.
4. Chillemi, F., Lugaro, G., Boari, D., Cardellini, E. *et al.* (1991) *FEBS Lett. 291*:67-70.
5. Cardellini, E., Bramucci, M. and Gianfranceschi, G.L. (1994) *Biol. Chem. Hoppe-Seyler 375*:255-259.
6. Angiolillo, A., Bramucci, M., Marsili, V., Panara, F. *et al.* (1993) *Mol. Cell. Biochem. 125*:65-72.

Analysis of the ATP/GTP Binding Site of Casein Kinase II by Site-Directed Mutagenesis

Rolf Jakobi and Jolinda A. Traugh

University of California, Riverside, CA, U.S.A. 92521

Casein kinase II is one of only a few protein kinases which effectively utilize ATP and GTP in the phosphotransferase reaction. A comparison of the catalytic domain of all protein kinases allows identification of highly conserved amino acids, a number of which are associated with the nucleotide binding site. In casein kinase II, two residues conserved in the ATP binding domain of other protein kinases are unique to the catalytic (α) subunit of casein kinase II. Val-66 is present in subdomain II and Trp-176 in subdomain VII, while >95% of the other protein kinases contain alanine and phenylalanine, respectively. The residues in the subunit of casein kinase II were changed to the conserved residues via single and double mutations by site-directed mutagenesis. These substitutions enhanced the utilization of ATP over GTP by altering the K_m values of the subunit for the ATP and/or GTP. Following reconstitution of the catalytic subunit with the regulatory (β) subunit, both the K_m and V_{max} of the reconstituted $\alpha_2 \beta_2$ holoenzyme are altered. A comparison of casein kinase II with the x-ray crystallographic structure of the catalytic subunit of cAMP-dependent protein kinase shows Val-66 and Trp-176 are part of the active site and in close proximity to each other. Val-66 is part of the β sheet and located two residues N-terminal to the invariant lysine which contacts the α and β phosphates of the nucleotide. The side chain of Val-66 is close to the purine ring of the nucleotide. Trp-176 is in the middle of two conserved residues, aspartic acid which chelates Mg^{+2} bound to the β and γ phosphates of the nucleotide, and glycine. The side chain of Trp-176 is part of a hydrophobic pocket which brings functional residues into appropriate orientations. Interestingly, the mutations are also shown to reduce or eliminate the 4- to 5-fold increase in catalytic activity observed with the holoenzyme over that of the subunit alone. Structural studies using UV circular dichroism spectroscopy show that effects of the mutations on the catalytic activity correlate with changes in the secondary structure of the holoenzyme. Taken together, the data indicate that utilization of both ATP and GTP can be directly correlated with stimulation of catalytic activity by the regulatory subunit and suggest a co-evolution of these separate functions.

Peptide-Based Immunomapping and Crosslinking Analysis of Subunit Contact Sites in Human Protein Kinase CK2 (Casein Kinase II) and Accessibility of Sites as a Means to Study Cellular CK2 Function

Walter Pyerin, Andreas Kreahn and Peter Lorenz

Biochemical Cell Physiology, German Cancer Research Center, D-69120 Heidelberg, Germany

Protein kinase CK2 is a heterotetramer composed of two catalytic (α and/or α′) and two regulatory (β) subunits. Subunit interaction by predominantly mutational analysis have indicated several domains that appear to be responsible for subunit interaction and enzyme control. We have been contributing to this problem by non-mutational methods including peptide-based immunomapping and chemical cross-linking analysis. As a result, one of the sites that appear to be involved in contacting is structure fragments 140–156 of subunit β (β140–156). Further, by employing antibodies raised against or neutralized by peptide β 140–156, evidence has been provided for the involvement of CK2 in signaling and early events in mitogenic stimulation of human cells in culture.

References

Lorenz, P., Pepperkok, R., Ansorge, W. and Pyerin, W. (1993) *Biol. Chem. 268:*2733–2737.

Wirkner, U., Voss, H., Lichter, P., Ansorge, W. and Pyerin, W. (1994) *Genomics 19:*258–265.

Bodenbach, L., Fauss, J., Rotitzki, A., Lozeman, F.J. and Pyerin, W. (1994) *Eur. J. Biochem. 220:*263–273.

Pepperkok, R., Lorenz, P., Ansorge, W. and Pyerin, W. (1994) *Biol. Chem. 269:*6986–6991.

Pyerin, W. (1994) *Advances in Enzyme Regulation*, Vol. 34, Pergamon Press, New York, pp. 225–246.

Characterization of Nucleolar Phosphoprotein p120

**E. Durban, Y. Ren, C.W. Taylor,
W.C. Gustafson, B. Valdez and H. Busch**

Department of Pharmacology, Baylor College of Medicine, Houston, Texas 77030

Nucleolar phosphoprotein p120 was originally identified exclusively in nucleoli of proliferating cells by a monoclonal antibody (Freeman *et al.*, 1988, *Cancer Res. 48:*1244–1251). Protein p120 is present in the nucleolus in relatively low amounts compared with other known nucleolar phosphoproteins such as protein C23 (nucleolin) and protein B23 (nucleophosmin). Both the amino acid sequence and genomic structure of protein p120 including upstream control regions have been determined as well as several functional domains (nuclear/nucleolar localization signal, casein II kinase phosphorylation site).

By sucrose gradient centrifugation, p120 sedimented as a major peak in the 60–80 S region in which pre-ribosomal particles sedimented including 28 S and 18 S RNA. Treatment of p120 with buffers of increasing ionic strength shifted the p120 sedimentation pattern towards the top of the gradient reflecting dissociation of p120 from the preribosomal particles. Complete dissociation was achieved with 1M KCl. A major fraction of p120 was also shifted towards the top of the gradient when nucleolar extracts were treated with RNase before sucrose gradient ultracentrifugation indicating that the association of p120 with the 60–80 S particles was dependent on interaction with RNA. DNase had no effect on p120 sedimentation behavior indicating no interaction of p120 with DNA.

Since studies on functional domains are facilitated by expression of p120-peptides, overexpression of p120 in the baculovirus/insect cell system was explored. About 20% of total protein in insect cells infected with recombinant virus was protein p120. Both by immunofluorescence and subcellular fractionation, recombinant protein p120 was mainly localized in nucleoli. When insect cells were incubated with P-32 orthophosphate, p120 could be identified as a phosphoprotein labeled *in vivo*.

The association of p120 with pre-ribosomal RNA suggested by these experiments is of particular interest considering the recently proposed function of p120 as a rRNA methylase (Koonin, 1994, *Nucleic Acids Res. 22:*2476–2478). Experiments are in progress to investigate this potential function of protein p120.

Phosphorylation of C-Terminal Domain of RNA Polymerase II is Required in Initiation of Productive Transcription of Balbiani Ring 2 Genes

Endre Egyházi, Amina Ossoinak, Andrew Pigon, Jae Moon Lee[1] and Arno L. Greenleaf[1]

Karolinska Institutet, CMB, Medical Cell Biology, Stockholm, Sweden and
[1]Department of Biochemistry, Duke University Medical Center, Durham, North Carolina, USA

Using polytene chromosomes of salivary gland cells of *Chironomus tentans*, phosphorylation state-sensitive antibodies and the transcription and the protein kinase CKII inhibitor 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB), we have visualized the chromosomal distribution of RNA polymerase II (pol II) with hypophosphorylated (pol IIA) and hyperphosphorylated (pol IIO) carboxyl-terminal repeat domain (CTD). DRB blocks labeling of the CTD with $^{32}P_i$ within minutes of its addition, and nuclear pol IIO is gradually converted to IIA; this conversion parallels the reduction in transcription of protein coding genes. DRB also alters the chromosomal distribution of IIO: there is a time-dependent clearance from chromosomes of phosphoCTD (PCTD) after addition of DRB which coincides in time with the completion and release of pre-initiated transcripts. The staining pattern of chromosomes with anti-CTD antibodies is not detectably influenced by the DRB treatment, indicating that hypophosphorylated pol IIA is unaffected by DRB. Microinjection of anti-PCTD IgG and anti-CTD IgG into living salivary gland cells inhibits the transcription of BR 2 genes. The treatment of salivary glands with DRB under heat shock conditions is without effect on the phosphorylation of CTD or the transcription of BR genes. The results demonstrate that under normal conditions *in vivo* the protein kinase effector DRB shows parallel effects on an early step in gene transcription and the process of pol II hyperphosphorylation. Our observations are consistent with the proposal that beginning productive RNA synthesis is CTD-phosphorylation dependent and also with the idea that the gradual dephosphorylation of transcribing IIO is coupled to the completion of pol II gene transcripts.

Use of Phage Peptide Libraries for Studying the Specificity of Tyrosine Phosphorylation and Recognition

Luciana Dente, Costantino Vetriani, Gianni Cesareni

Department of Biology, "Tor Vergata," Rome, Italy

Phosphorylation is one of the most important posttranslational modifications which regulate signal transduction. Following a variety of hormonal or mitogenic stimuli, signals are transduced from the cell surface to the nucleus through a cascade of protein phosphorylations and protein-protein interactions.

Two classes of protein kinases (serine/threonine and tyrosine kinases) and specific protein domains (SH2, SH3, PH) are involved in these processes. In particular, phosphorylation of tyrosine and its direct interaction, on the basis of the surrounding aminoacids, with specific SH2 domains are crucial steps in the early phase of signal transduction.

Phage display technology can be exploited to study the specificity of the process of phosphorylation. We are using phage peptide libraries for identifying the substrate of specific tyr-kinases, either purified or in a cell extract. Phages displaying peptides can be phosphorylated *in vitro* and selected by affinity purification with anti-phosphotyrosine antibodies. The sequences of the displayed peptides are then deduced from the nucleotide sequence of the corresponding hybrid genes.

Furthermore, we have selected classes of phosphotyrosine peptides which bind specific SH2 domains (purified by the group of P. Pelicci), using phage libraries, modified by extensive phosphorylation. In such a library all the tyrosines are phosphorylated, irrespective of their structural context.

Analysis of different Tyrosine-kinases, different SH2 domains and identification of their specific substrates should contribute to the identification of the elements that determine the specificity of these interactions.

ATP/GTP-Dependent Phosphorylation of P-Glycoprotein, the Transporter Responsible for Multidrug Resistance

I.H. Lelong - UPR 9003 DU CNRS

IRCAD-Hopitaux Universitaires-BP 426-F67091 Strasbourg Cedex, France

Numerous peptides and proteins undergo structural modifications by phosphorylation-dephosphorylation process(es). These processes operate usually as a key regulatory element for protein activities and often involve a complex interplay of protein kinase(s) and phosphoprotein phosphatase(s). The intervention of several nucleotides capable of modulating the protein phosphorylation may even render these phenomenon more complex. In our studies focused on the structure/function of P-glycoprotein, the transporter responsible for multidrug resistance (MDR), we have observed strong phosphorylation level modulations by ATP/GTP combinations in the [γ-^{32}P]ATP mediated phosphorylation of the protein. The model that we have used for our phosphorylation studies consists of an *in vitro* system of sealed plasma membrane vesicles isolated from KB-V1 human cancer cells. These cells present a pleiotropic resistance towards numerous chemo-therapeutic agents which is correlated by the overexpression of P-glycoprotein. The plasma membrane vesicles system from KB-V1 cells enables ^{3}H-vinblastine accumulation studies as a measure of the transport activity of P-glycoprotein and permits phosphorylation studies of the protein as well. In this system, the ^{3}H-vinblastine transport was shown to be ATP-dependent. Furthermore, we have also shown that GTP could replace ATP as the energy source to drive the transport of ^{3}H-vinblastine while neither UTP nor CTP could sustain it. With this system, using [γ-^{32}P]ATP we have shown that the transporter is endogenously phosphorylatable in the absence of any exogenously added protein kinases. This phosphorylation was not diminished in the presence of the specific inhibitors of the well known "classical" protein kinases PKC and PKA. We have also shown that the level of [γ-^{32}P]ATP driven P-glycoprotein phosphorylation can be modulated by GTP since different combinations of GTP and ATP result in differential phosphorylation levels of the multidrug transporter by [γ-^{32}P]ATP. Tryptic [γ-^{32}P]phosphopeptide maps deriving from P-glycoprotein phosphorylated by [γ-^{32}P]ATP show that addition of GTP alters relative labeling of phosphopeptides. Additional studies have shown that the transporter can be phosphorylated using [γ-^{32}P]GTP as the phosphate donor and that in this case ATP/GTP combinations also triggered modulations in phosphorylation levels. Our results suggest that the overall phosphorylation of P-glycoprotein is determined by several protein kinases and phosphatases, at least one of which may be GTP-regulated. To understand the mechanism of transport of P-glycoprotein, studies are now undertaken in order to determine the incidence of phosphorylation levels of the transporter on its transport capacity and vice versa.

Effects of the Phorbol Ester TPA and 1-OLEOYL-2-Acetyl Glycerol on the β-Hexosaminidases of HL 60 Cells

C. Emiliani, S. Martino, J.L. Stirling[1] and A. Orlacchio

Dipartimento di Biologia Cellulare e Molecolare, Sezione di Biochimica e Biologia Molecolare, Università degli Studi di Perugia, via del Giochetto, 06126 Perugia, Italy and
[1]Division of Life Sciences, King's College London, Campden Hill, London W87AH U.K.

In normal human tissues the lysosomal enzyme β-N-acetylhexosaminidase (Hex, E.C. 3.2.1.52) exists as two major forms A (αβ) and B (ββ). Proportions of the two forms vary somewhat in different tissues but generally occur in proportions of: A 60-70%; B 30-40%. Very little, if any, αα dimer is seen in normal tissues, but in Sandhoff's disease, in which the production of functional β subunits is affected, small amounts of Hex S (αα) are observed.

Many leukaemic cells lines have aberrant Hex profiles (1). In the human leukaemic cell line HL 60, while the B form is reduced to 5% of total activity, there is a greatly increased amount of Hex S (2). Additionally, some Hex activity is associated with the plasma membrane of HL 60 cells (3). HL 60 cells can be made to differentiate along the granulocyte pathway by treatment with DMSO; we have shown that this is accompanied by decreased production of Hex S and increased formation of Hex B (4). The resulting Hex profile is one more closely resembling that seen in normal granulocytes. Treatment of HL 60 cells with the phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA) causes differentiation along the macrophage pathway. TPA treatment of HL 60 cells affects the Hex profile in different ways depending on the length of exposure. There were transient effects observed after up to 24 hours of exposure (short term effects) that included a basic shift of the A form, an acidic shift of S and increased abundance of B as well as the appearance of an additional acidic form. Transient effects were followed by longer term effects (48-72 hr) which restored isoenzyme profiles to those seen in fully differentiated cells. In order to elucidate to what extent short term change were due to a direct effect of TPA on stimulation of protein kinase C (PKC), we treated HL 60 cells with a more specific activator of PKC, 1-oleoyl-2-acetyl glycerol (OAG), which does not cause cell differentiation. OAG was shown to have even greater short term effects on the Hex isoenzyme profile than TPA. Studies of subcellular fractionation demonstrated that the greatest short term effects of TPA and OAG were on the Hex isoenzymes associated with the plasma membrane of HL 60 cells. Thus, we deduce that PKC might have a direct effect on the subunits of Hex, and that the effect of TPA on the enzyme likely parallels, rather than arises as a consequence of, the induction of differentiation. Analysis of the α- and β-subunit sequences revealed that they contain a number of motifs that could act as substrates for protein kinase C. Work is in progress to ascertain if they may function as phosphorylation sites *in vivo*.

Work supported by Italian CNR, Progetto Finalizato A.C.R.O., SP 4, contract #94.02208 PF39 and Progetto Finalizzato Ingegneria Genetica, contract # 93.000.32.

References

1. Emiliani, C. and Oriacchio, A. (1992) *Int. J. Biochem. (Minireview) 24:*539-544.
2. Emiliani, C., Beccari, T., Tabilio, A., Ortacchio, A., Hosseini, R. and Stirling, J.L. (1990) *Biochem. J. 267:*111-117.
3. S. Martino, S., Emiliani, C., Orlacchio, A., Hosseini, R. and Stirling, J.L. (1994) *Biochim. Biophys. Acta*, in press.
4. Emiliani, C., Falzetti, F., Orlacchio, A. and Stirling, J.L. (1990) *Biochem. J. 272:*211-215.

Intermediate Filaments and Gene Regulation

Peter Traub

Max-Planck-Institut für Zellbiologie,
68526 Ladenburg/Heidelberg, Germany

Although intermediate filaments (IFs) are widely distributed in nature, their biological role is still largely unknown. They are constructed from a multitude of evolutionarily conserved, developmentally regulated and cell type-specific subunit proteins, implying that they are functional in differentiation processes. On the basis of their cytoplasmic distribution, relatively low dynamics, mechanical stability and interaction with a variety of subcellular structures, they are believed to act as mechanical integrators of intracellular space. However, genetic experiments, e.g. knock out of individual IF proteins in transgenic mice, have demonstrated that IFs as such are not as essential for cellular activities as hitherto presumed and that their subunit proteins must therefore exert functions going beyond a merely cytoskeletal role.

Our own studies in this direction have revealed that cytoplasmic (c) IF proteins are nucleic acid-binding proteins with high affinities for guanine(g)-rich DNAs as well as for supercoiled DNA. They also interact in a distinct stoichiometry with core histones. While the former activity is mediated by their structurally variable, non-α-helical, but commonly arg-rich N-terminal head domains, the latter is restricted to their conserved α-helical rod domains. Structurally, cIF proteins show close parallels with gene-regulatory DNA-binding proteins in that they possess a central, α-helical dimerization motif flanked by a positively charged DNA-binding site. cIF proteins also resemble nuclear matrix proteins which are frequently constructed from long α-helical regions in close proximity to basic, non-α-helical domains. A special class of nuclear matrix proteins, the nuclear lamins, are in fact genuine IF proteins.

On the basis of these properties and parallels, we propose that cIFs, although their subunits do not possess nuclear localization signals, also fulfill nuclear functions. This conjecture is supported by the enrichment of cIFs around the nucleus, their non-ionic detergent- and high salt-resistant association with the nuclear periphery, and distortion of the double nuclear membrane at cIF-nucleus contact sites. To penetrate the perinuclear cisternae, the cIFs probably make use of the amphiphilic character of the N-terminal head domains which, together with the C-terminal tail regions, are repetitively arranged on the surface of the filament body proper. The same arrangement might be exploited by the cIFs to interact with repetitive elements of the nuclear matrix and/or peripheral chromatin. In this way, the cell type-specific cIFs might participate in the non-random distribution of chromosomes and organization of chromatin in the interphase nuclei of differentiated cells and thus in the regulation of gene expression or other DNA-based nuclear events.

In order to provide evidence in favor of this notion, we have isolated a collection of DNA sequences by several rounds of affinity binding of a mixture of genomic mouse DNA fragments to different types of *in vitro* reconstituted cIFs and PCR amplification of the bound fragments. The fragments were, as expected, rich in G and highly repetitive and structurally similar to regulatory sequence elements in the 5′/3′-flanking regions and introns of genes. In addition to their capacity to adopt non-B DNA conformations under superhelical strain, most of the fragments contained one to several consensus binding sites for known transcription factors and recombination-active proteins. The different cIF types bound conceptionally similar repetitive sequence elements, yet these sequences were generally different for the various cIF types. In addition, the cIFs also selected with high efficiency centromeric γ-satellite DNA sequences from the fragment mixture. Since vimentin also binds with high affinity to

repetitive oligonucleotide telomere models, we interpret these results to indicate that cIFs may also be involved in position effects associated with translocation of genes to heterochromatic centromere and telomere regions of the chromosomes, the repeat sequences of which are often localized to the nuclear periphery in cell type-specific, non-random patterns.

Protein-DNA Interactions at the Nuclear Scaffold Attachment Regions of DNA Loops

Anna Ferraro, Margherita Eufemi, Laura Cervoni and Carlo Turano

Dipartimento di Scienze Biochimiche 'Alessandro Rossi Fanelli' e Centro di Biologia Molecolare del CNR, Università La Sapienza, Roma, Italy

DNA in cell nuclei is organized in large loops, formed by the binding of the scaffold attachment regions (SARs) of DNA to some proteins present in the internal nuclear scaffold (or matrix) and in the peripheral nuclear lamina. It is nowadays recognized that these regions have not only a structural role, but also a functional one, since they define transcriptional domains of the chromatin, and contain enhancer regions and probably replication origin regions as well. The identification and characterization of the proteins responsible of the binding of SARs is therefore an important task, and in fact more than thirty of them have so far been described. However, the identification of these proteins and of the SARs can in principle be questioned, since the nuclear scaffold structure itself is strongly dependent on the method of preparation, and the scaffold may even be undetectable (except for the nuclear lamina) if some procedure for nuclei stabilization is not performed.

In order to avoid these problems, we have analyzed the DNA-proteins interactions directly in the intact nucleus by means of cross-linking reagents. The proteins isolated from the complexes formed in this way have then been isolated and compared with the SAR-binding proteins so far described. Most of the protein species isolated from the complexes appear to derive from the nuclear scaffold (prepared by the usual procedures) and, by means of South-Western blotting, have been shown to recombine specifically with DNA. While a subset of these proteins recognizes SAR-sequences and the related poly dA-poly dT polynucleotide, another subset binds specifically other, still uncharacterized, DNA sequences.

By the same method, the nuclear scaffold proteins interacting with DNA in chicken liver nuclei and erythrocyte nuclei have been compared. It has been found that different proteins are involved in the interactions in the two cases. Those from erythrocytes are represented by only three-four species with a low molecular weight, all derived from the nuclei periphery, while those from liver are represented by many species with molecular weights ranging from 30 to 120 kDa, and derive from the internal and from the peripheral scaffold. These differences are likely to be correlated with the difference in transcriptional activity of the two tissues examined. These results and the potentiality of this approach will be discussed, particularly in relation to the results obtained by the conventional procedures.

This work was supported by grants from Ministero del'Università e della Ricerca Scientifica e Tecnologica.

A Family of Proteins with Centrosomal and Nuclear Localization

Antonella Angiolillo[1], Gaby Joswig[2] and Dieter Werner[2]

[1]Department of Cellular and Molecular Biology, University of Perugia, Italy and
[2]German Cancer Research Center, Division of Biochemistry of the Cell, Heidelberg, Germany

In an attempt to identify structural non-histone components of the nucleus, a number of laboratories have extracted nuclei using various combinations of salts, detergents and nucleases. The remaining insoluble residue has been termed the nuclear matrix or nucleoskeleton.

Most nuclear matrix preparations contain a filamentous structure that varies greatly in appearance and composition depending on the procedure used to isolate it. Due to this variability and of the possibility of aggregation-induced artifacts, the *in vivo* existence of the nuclear matrix has been often put into question.

However, despite its controversial status, the nuclear matrix has been suggested to be the site of a number of critical nuclear functions, including DNA replication, transcription, hnRNA processing, gene expression and maintenance of higher order chromatin structure.

It is widely assumed that chromatin is looped by attachment to the nucleoskeleton. These attachments seem to be mediated through specific sequences, and cell types of different tissues (or even cells of the same tissue) would have different attachments of this type, resulting in the tissue specific regulation of genes.

At present the best characterized component of the nuclear matrix is the nuclear lamina, which is composed of proteins related to intermediate filament proteins. The nuclear matrix which extends into the interior of the nucleus seems to be composed of similarly fibrous components. Several nuclear matrix proteins with filamentous structure have been already characterized. It was observed that some of them relocate from the interphase nucleus to the centrosome at prophase and accumulate at the spindle poles in metaphase and anaphase. During telophase they relocate to the reassembling nucleus.

In this work a new family of proteins with centrosomal and nuclear locations has been identified. Monoclonal antibodies were prepared against the residual material of deproteinized and DNasI-digested DNA. The antibodies were used to screen a λgt11 murine RNA-based library. A positive clone was plaque purified (I. Batova, Sofia). A fragment of this initial clone was used to screen a λgt10 library. Three different families of clones were identified, which hybridized with the probe. The opening reading frame of one of these clones, λIB3/5, was sequenced, it consists of 3.2kB and codes for a protein of ~760000Da. The nucleotide and aminoacid sequences were not previously described. Appropriate computer programs were employed to predict long coiled-coil regions.

Segments of the coding region of IB3/5 were subcloned in an expression vector and antibodies against the recombinant antigen were prepared. They were used for immunofluorescence studies to demonstrate the location of the proteins. The results indicate that the antigen is localized both in the nucleus and the centrosome.

High-Salt and SDS-Stable DNA-Binding Protein Complexes with ATPase and Protein Kinase Activity Retained in Chromatin-Depleted Nuclei

Benediktas Juodka[3], Eberhard Spiess[2], Antonella Angiolillo[4], Gaby Joswig[1], Karsten Rothbarth[1] and Dieter Werner[1]

[1]German Cancer Research Center, Division Biochemistry of the Cell (0225) and
[2]Biomedical Ultrastructure Research Unit (0195), D-69009 Heidelberg, Germany,
[3]Department of Biochemistry and Biophysics, Vilnius University, Vilnius, Lithuania, and
[4]Department of Cell and Molecular Biology, University of Perugia, Perugia, Italy.

DNA-polypeptide complexes with higher stabilities than nucleosomes are considered to be involved in the topological organization of DNA and in tissue-specific regulation of gene expression. Approaches to isolate and to characterize nonhistimine polypeptides involved in this function are usually based on extractions of nuclei with agents that release soluble proteins and the bulk of chromatin which is then followed by investigation of the residual nuclear components. One alternative approach to identify tight structures between polypeptides and genomic DNA is based on the assumption that relevant DNA-polypeptide complexes may exist which are more stable than the basic nuclear structure itself. In this case isolated DNA should remain associated with residual polypeptides that are not released by methods sufficient to disrupt the integrity of the basic nuclear structure. Here we show that a rapid and simple deproteinization method allows a yield of tight DNA-protein complexes which show the characteristics of anchorage complexes.

Cell lysis in presence of SDS and proteinase K followed by salting out of residual polypeptides by dehydration and precipitation with saturated sodium chloride solution resolves efficiently deproteinized DNA. However, this DNA is still associated with prominent polypeptides which remain stably attached to DNA during further treatments, e.g. during repeated salting out steps, prolonged incubation of DNA in 1% SDS or 4 M urea at 56°C and during ethanol precipitations. The persistent polypeptides (62 kDa, 52 kDa and 40 kDa) released from Ehrlich ascites cell DNA were further characterized. Microsequencing indicates that the DNA-binding polypeptides are not yet characterized on the sequence level. Nuclease digestion of the DNA releases stable DNA-protein complexes with the shape of globular particles (12.8 ± 0.8 nm) and their larger aggregates in which DNA remains protected from nuclease digestion. The isolated DNA-polypeptide complexes show ATPase (K_m = 7.4×10^{-4} M) and protein kinase activity. Antibodies reveal a parallel distribution of the complexes with chromatin, however, the complexes are retained in chromatin-depleted nuclei.

Short DNA Sequences Tightly Associated with Proteins in the Cytoplasm of Tumor Cells

Ralf Hegger and Hinrich Abken

Klinik I für Innere Medizin, Labor Tumorgenetik, Universität zu Köln, D-50924, Köln, Germany

Previously, we reported (1) that the mouse L929 and Ehrlich ascites tumor cells harbor cytoplasmic DNA sequences that induce unlimited proliferation of human lymphocytes after transfection *in vitro*. The DNA was recovered from extra-mitochondrial fractions of the cytoplasm by equilibrium centrifugation in a neutral CsCl gradient. These fractions contain linear DNA molecules of 50-500 bp in length. Unexpectedly, the short linear DNA sequences were found tightly bound to at least three proteins (52 kD, 62 kD, 64 kD). The proteins remain stably bound to DNA after treatment with proteinase K, in the presence of high salt concentrations (2M NaCl), after incubation with guanidine-HCl (6M) or NaOH (0.1 M), and after extraction of the DNA with phenol and chloroform. We conclude that the linear DNA sequences persist as salt- and proteinase-stable DNA-protein (DNP) complexes within the cytoplasm of the tumor cells. Hydrolysis of the DNA by DNase I makes the DNP complex accessible for protein degradation by proteinase K impying that the DNA may be arranged on the outside surface of the DNP complex. Although the organization of the cyto-plasmic DNA-protein complex seems to be similar to that of nucleosomes, two major points distinguish the DNP complex from nucleosomes: (i) the proteins of the DNP complex do not react with anti-histone antibodies, (ii) the DNA protein interaction is even more salt- and detergent-stable than that of nucleosomes. This type of DNA-protein interaction was reported for chromosomal DNA by Juodka *et al.*, 1991, (2) who showed that certain nuclear DNA sequences are tightly bound to proteins of the nuclear matrix by covalent phosphotriester bonds between hydroxylgroups of the amino acids and the internucleotide phosphate group of the DNA. More recent experiments suggest that the proteins bound to the cytoplasmic DNA sequence are related to those nucleomatrix proteins. The protein associated cytoplasmic DNA sequences however, are not identical to those nuclear sequences associated with nuclear matrix proteins. The functional relevance of the cytoplasmic DNP complexes in tumor cells is currently under investigation.

References

1. Abken, H., Hegger, R., Bützler, C. and Willecke, K. (1993) *Proc. Natl. Acad. Sci. USA 90:*6518-6522.
2. Juodka, B., Pfütz, M. and Werner, D. (1991) *Nucl. Acids. Res. 19:*6391-6398.

Structural Determinants of the Interaction Between the RNP-1 Consensus Sequence and Single-Stranded RNAs

Monique Erard[1], Abdelhakim Kharrat[1], Honoré Mazarguil[2], Philippe Bouvet[1] and François Amalric[1]

[1]Laboratoire de Biologie Moléculaire Eucaryote, 118 Route de Narbonne, 31062 Toulouse Cédex, France and [2]Laboratoire de Pharmacologie et Toxicologie Fondamentale, 205 Route de Narbonne, 31077 Toulouse Cédex, France

It is likely that all the RNA recognition motifs (RRMs) so far identified share the same folding pattern ($\beta_1\alpha_1\beta_2\alpha_2\beta_4$), which is based on the distribution of conserved hydrophobic residues throughout the 90-amino acid domain. The most conserved element is the RNP-1 consensus sequence. This octapeptide comprises the 7 residue β_3 strand plus the conserved N-terminal basic residue. It is likely that the RNP-1 sequence mediates the initial contact with RNA, prior to the formation of a more specific interaction. In order to investigate the RNA binding properties of this sequence, we have synthesized the peptide pRRMc (GKSKGFGFVEFKSE), corresponding to the RNP-1 sequence (in bold characters) and 6 flanking residues. Based on the fact that the only apparent similarity shared by the various RNAs which interact with RRMs is a number of unpaired ribonucleotide bases, we have probed the interaction between pRRMc and various single-stranded polyribonucleotides by using Fourier transform infrared spectroscopy and circular dichroism. These experiments provide evidence that the RNP-1 consensus sequence interacts directly with single-stranded RNA. Changes in the RNP-1 β-strand structure and a consequent reorientation of the conserved valine residue have been revealed by infrared spectroscopy. The RNA-peptide interaction appears to involve both the stacking of phenylalanine residues with nucleic bases and ionic contacts between lysine residues and the sugar-phosphate backbone.

ARTICLES

Physiol. Chem. Phys. & Med. NMR (1995) 27:271-280

Selection of Phage-Displayed Peptides Mimicking an Extracellular Epitope of Human MDR1-P-Glycoprotein

Francesca Poloni[1], Sabrina Palumbo[1], Maurizio Cianfriglia[1] and Franco Felici [2]

[1]Laboratorio di Immunologia, Istituto Superiore di Sanità, viale R. Elena 299, 00161 Roma, Italy and [2]IRBM (Istituto di Ricerche di Biologia Molecolare P. Angeletti), via Pontina Km 30.600, 00040 Pomezia, Roma, Italy

Abstract: To study the structural conformation of the MM4.17 monoclonal antibody (mAb) epitope, twenty-six mAb MM4.17-specific phage clones were affinity-isolated and their inserts characterized for aminoacid composition and homology with MDR1 gene product (MDR1-P-glycoprotein). The resulting sequence alignment shows that a unique consensus sequence, which corresponds to the previously mapped TRIDDPET linear peptide identified through synthetic peptide scanning, could not be identified. However, similarities between the inserts of positive phage clones and P-glycoprotein primary structure, consisting in two or three aminoacid-long sequences, were observed. An analysis of the over-represented aminoacid residues in the inserts of positive clones, and their comparison with the sequence of the antigen was also performed. The two different procedures led to the identification of four regions in which these similarities are clustered, indicating that four different antigen regions, one of which includes the TRIDDPET linear aminoacid sequence, might participate in forming the structure of monoclonal antibody MM4.17 epitope.

THE INHERENT or acquired expression of an effective drug transport system is the principal means by which tumor cells can actively proliferate in the presence of structurally and functionally diverse cytotoxic compounds (1-3). Such multiple drug resistance (MDR) is one of the most challenging topics in medicine since it represents a serious obstacle to successful chemotherapy of tumors (4-5), diseases produced by protozoan parasites (6) or infections by pathogenic fungi (7) and yeasts (8). In humans, this form of MDR is usually mediated by the over-expression of MDR1 gene-encoded class-I P-glycoprotein (P-glycoprotein) which acts as an ATP-driven drug-efflux pump, decreasing intracellular anti-cancer drugs bio-availability (9).

The secondary structure predicted for P-glycoprotein, derived from the analysis of its

aminoacid sequence, includes 12-transmembrane segments and two large cytoplasmic domains that contain two ATP-binding consensus sequences (10). The Eisenberg hydropathy analysis (11) of the P-glycoprotein aminoacid sequence predicts six small regions of the molecule being exposed on the extracellular side of the plasma membrane (10). Mapping specific monoclonal antibodies (mAbs) epitopes, could help in confirming the above topographical model of P-glycoprotein, and hence in elucidating the relationships between its structural organization and drug-efflux pump function.

Previous studies using mAbs C219 and C494 (12) which stain only permeabilized mammalian MDR cells have demonstrated that two large hydrophilic P-glycoprotein domains are located on the cytoplasmic side of the plasma membrane, while synthetic peptide scanning of mAbs MRK-16 (13), MM4.17 (14), and MM6.15 (15) directed against live human MDR cells demonstrated that particular regions of the predicted first, fourth and sixth P-glycoprotein extracellular regions are indeed external. In particular, the results obtained from mAb MM6.15 epitope mapping studies suggest that the above three extracellular loops are close in the folded structure, forming a single antibody epitope (13,15). Sometimes mapping of P-glycoprotein epitopes through peptide scanning may be, however, seriously limited by partial reconstitution of protein domains, or by epitope structures not being totally reproducible using linear synthetic peptides. The mAb MC57 epitope, for example, which is extracelluar and more efficiently expressed on inactivated forms of P-glycoprotein molecules (i.e. after reversing agent treatments of MDR cells (16)), could not be identified using peptide scanning. To map this antibody epitope we have recently utilized the phage display technology (17,18); surprisingly, among the P-glycoprotein regions identified as a putative part of the mAb MC57 epitope, a segment defined by residues 800-807 of the sequence, postulated to be intracellular, was included (19). This result represents an additional challenge to the classical 12-transmembrane domain model of P-glycoprotein, since it agrees with the novel topography of the molecule recently proposed on the basis of biochemical (20) and expression (21) studies.

The result obtained from MC57 epitope mapping through screening of phage-displayed peptide libraries, prompted us to use this technology to analyze other P-glycoprotein epitopes, including those already studied through synthetic peptide scanning, with the aim of exploring the potentialities of these different techniques in the analysis of structural organization of P-glycoprotein. The results we obtained suggest that the MM4.17 epitope, previously mapped in the TRIDDPET peptide sequence and hence localized in the fourth extracellular loop (14), could actually be larger, as they identify three more regions of P-glycoprotein as putative constituents of this epitope.

Materials and Methods

Monoclonal antibodies (mAbs) and cells

MM4.17 (IgG2a,k) (14) was developed in mice through immunization with cells from an MDR variant of the human T-lymphoblastoid CCRF-CEM leukemia cell line. This mAb is P-glycoprotein specific, as it stains living rodent cells transfected with human MDR1 gene and reacts only with inter-specific cell hybrids (preferentially retaining the human chromosome 7 and the resistant phenotype), obtained through somatic fusion of sensitive rodent cells with human MDR cells.

MC57 (IgG2a,k) (22) was also raised in mice against the MDR variant of living CEM cells. Its reactivity with MDR1 gene-transfected rodent cells, and also with MDR inter-specific cell hybrids, confirms its specificity for MDR1-gene product.

These two mAbs were utilized for cell staining in purified form at a concentration of 12.5 μg/ml. A human T-cell system represented by the parental drug-sensitive CCRF-CEM(CEM) cell and its CEM-VBL100 MDR variant were routinely used to control the specificity of the antibodies towards MDR1-P-glycoprotein. The cell culture conditions were according to standard protocols for cells growing in suspension, including RPMI-I640 medium enriched by 5% of Fetal Bovine Serum (HyClone Laboratories, Inc. Logan, Utah) and antibiotics.

P-glycoprotein expression on cells

Flow-cytometry analysis was performed by standard procedures, revealing mAb binding by a FITC-conjugated F(ab')2 goat anti-mouse IgG (GAM, Cappel, West Chester, PA). After staining, cells were fixed in 1% formaldehyde in PBS, pH 7.2, and analyzed on a bench-top flow cytometer (FACScan, Becton Dickinson, Mountain View, CA) equipped with a 15-mW argon ion laser emitting light at a fixed wavelength of 488 nm. Fluorescence signals were collected in logarithmic mode (4-decade logarithmic amplifier) and relative cell numbers per channel in linear mode. In all tests, negative controls included non-related mAbs. To visualize mAb reactivity, the cells mounted in glycerol were examined with a Zeiss RA microscope equipped with epifluorescence optics.

Affinity selection of phage peptide libraries

The phage peptide libraries used in this study (pVIII-9aa (23); pVIII-9aa.Cys (24)) contain 9 aminoacid random peptide inserts in the N-terminal region of the phage major coat protein (pVIII); in one of the two libraries used for selection (pVIII-9aa.Cys), the random inserts are flanked by two cysteines residues and hence are cyclically constrained. Specific phage clones for mAb MM4.17 were isolated from both libraries by two rounds of affinity selection, according to previously described biopanning procedures (25, 23).

In the first one, the mAb (at 1 μM concentration) was incubated overnight at +4ºC with 10^{10} Amp^R TU of library in a total volume of 10 μl. The mixture was incubated with 0.25 μg of a biotin-conjugated goat anti-mouse IgG secondary antibody (Fc specific, SIGMA, St. Louis, MO), which was previously pre-adsorbed overnight at +4ºC with 2×10^{11} phage particles of UV-killed M13K07 in order to prevent unspecific binding, and then the phage-mAb-secondary Ab complexes were tethered on streptavidin coated dishes. The selected phage clones were then amplified through infection of XL1-blue bacterial cells, selection on Amp plates, and superinfection with M13K07 helper phage. The second round was carried out under stringent conditions (0.1 nM concentration of mAb was used), in order to select stronger reacting phage clones.

Immunoscreening of positive clones

Phage-producing colonies, each derived from a single phage clone, were obtained by infecting 0.4 ml of an overnight culture of XL1-blue bacterial cells with 10^4 Amp^R TU of the affinity-selected phage mixture, superinfecting with 10^{11} particles of M13K07 helper phage, and plating on 50 μg Amp/ml, 10 μg Kan/ml LB agar plates. Positive phage clones were identified through phage-colony immunoblotting (26). Positive clones were further

characterized through dot immunoblotting (23) and ELISA (27), using a 1 μg/ml concentration of mAb.

DNA sequencing

Single-stranded DNA was extracted from positive phage clones, and the sequence of the gene VIII 5′ region of the phagemid containing the 27 nucleotide long random insert was obtained. DNA sequencing was performed by the chain termination method (28) using the Sequenase version 2.0 kit (USB, Cleveland, OH). Translation of the DNA sequence allowed the corresponding specific peptide insert in the recombinant VIII protein to be defined.

Competitive inhibition assay

Increasing amounts of CsCl-purified phage clones (from 10^9 to 10^{13} phage particles/ml) were incubated for 3 hours at room temperature with 2 μg/ml of mAb MM4.17; the mixture was added to around 5×10^5 CEM-VBL100 cells and incubated 30 min at +4ºC. Cell suspension was then washed in PBS 1X and reacted with a FITC-conjugated F(ab′)2 goat anti-mouse secondary antibody (Cappel, West Chester, PA) for 30 min at +4ºC. After another washing step, the cells were analyzed for their fluorescence intensity by a FACScan cytometer (Benton Dickinson, West Chester, PA).

Construction of f-pep phage clone

The synthetic oligonucleotides (100 picomoles each) 5′ AATTCACCCGTATCGACGACCCCGGAAACCAAA 3′ and 5′ GATCTTTGGTTTCCGGGTCGTCGATACGGGTG 3′ were annealed at 65ºC for 10 minutes in SOH buffer (40 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 50 mM NaCl), and the mixture was allowed to cool at room temperature. Six picomoles of the resulting double-stranded oligonucleotide were ligated to 2 μg of EcoRI/BamHI digested pC89 (23) phagemid vector. E. coli XL1-blue cells were transformed with the ligation product and the relevant phage supernatant was prepared essentially as described (23). The predicted sequence of the phagemid-encoded protein is therefore NH_2-AEGEFTRIDDPETKDPAKAAFDSLQASATEYIGYAWAMVVVIVGATIGIKL FKKFTSKAS-COOH, containing the sequence TRIDDPET in the N-terminal region of the recombinant pVIII protein.

Results and Discussion

MM4.17 mAb was utilized in three independent selection experiments: using the pVIII-9aa library or the pVIII-9aa.Cys library separately, or an equal mixture of both libraries. After the first round of biopanning the phage recovery was, in all three cases, about 10^7 TU. A lower amount of phage (10^4 TU) was obtained after the second, more stringent, cycle. Phage-colony immunoblotting of clones deriving from the second round of selection revealed a percentage of positive colonies higher than 90%; 91 of them were randomly chosen for further analysis and the relevant phage supernatants were tested through dot-blot and ELISA. In both the above tests, the anti-mouse IgG secondary antibody alone and the mAb MC57, used as negative controls, proved non reactive to the selected clones (data not shown). We have considered as positives (significantly reacting) all the clones giving a signal in ELISA at least twice that of pC88 control phage (expressing wild-type pVIII); in

[a] clone	[b] insert sequence	[c] spot	[d] ELISA	[e] stdev
35	QPQNMISPR	+ / -	0.543	0.022
56c	GGHPVDMIS	+ + +	1.759	0.085
147	HVSEMVRPA	+	0.247	0.003
153	RICEFVPCS	+	0.347	0.039
128	PLPEMIAPV	+ / -	0.159	0.011
129	EEHEMIAPS	+	0.844	0.047
55	WHESNMIAG	+ + +	2.179	0.011
156c	YMIDSPL	+	0.177	0.011
57	LRMIESPIR	+ / -	0.376	0.050
32	LRMISPGEE	+ +	1.446	0.074
222	VSFMVQNTE	+ / -	0.189	0.007
52	PYMIPNAHS	+	0.536	0.016
155	ETWMVRPSG	+	0.301	0.021
37	PSFMVTPEA	+ +	1.455	0.046
226	ESWMINARQ	+ +	1.030	0.045
38	SDFRAFMVA	+ +	1.811	0.033
200	MPANVDPVM	+	0.332	0.014
223	MAADIDPAT	+	0.365	0.015
127	LPANIVRTA	+ +	0.467	0.034
58	PPADIATEA	+	0.509	0.081
144c	VDPPSAPSM	+ / -	0.280	0.021
220c	IDPPGVREA	+	0.358	0.013
40	IYADMIRAP	+	0.976	0.007
133	DPVKLMLDE	+ / -	0.405	0.031
39	DRGPILLSE	+ / -	0.430	0.013
152c	RGLPGDPIF	+ +	0.837	0.036
pC88	*(wild type)*	-	0.066	0.005
f-pep	TRIDDPETK	-	0.083	0.001

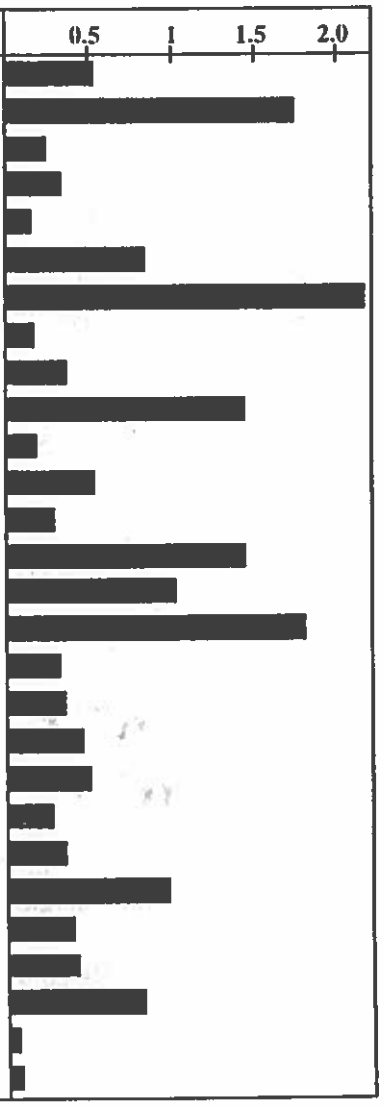


FIGURE 1. List of positive clones selected by the mAb MM4.17 from the two phage peptide libraries:

a. The clones containing a "c" after the identification number derive from the pVIII-9aa.Cys library, thus containing two cysteine residues flanking the insert; pC88 (expressing wild type pVIII of f1 bacteriophage) is used as negative control, and f-pep is a recombinant clone (not selected from the library) containing the TRIDDPET peptide sequence (see text).

b. For each clone, the aminoacid sequence of the insert is reported; this sequence is preceded by NH_2-AEGEF(C) and followed by (CG)DPAKAAFDSLQASATEYIGYAWAMVVVIVGA-TIGIKLFKKFTSKAS-COOH in the mature pVIII protein (the residues in parenthesis are present only in the pVIII-9aa.Cys library clones).

c. Semi-quantitative evaluation of the intensity of immuno-staining of phage spots on a nitrocellulose membrane.

d. The average values (*A* 405 nm) from two independent experiments are indicated both in numerical and graphical format (right panel).

e. The standard deviation of each pair of measures is reported.

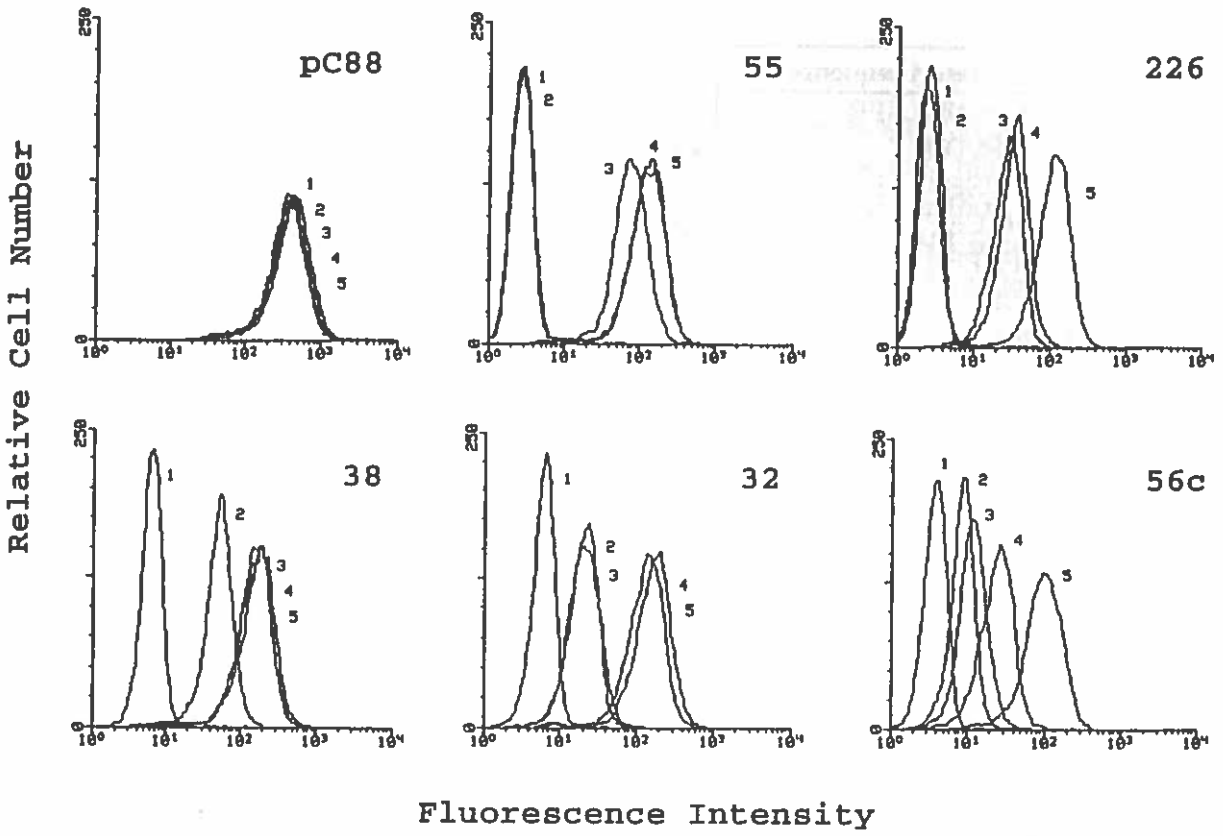


FIGURE 2. Inhibition of mAb MM4.17 binding to MDR variants of CEM cells. 50 µl of mAb MM4.17 (2 µg/ml immunoglobulin concentration) were pre-incubated with Log 10 serial dilutions of phage preparations (1 through 5) and then assayed for MDR cell staining. A direct correlation between phage concentration and staining inhibition, and the inability of wild type phage particles (pC88) to absorb MM4.17 immunoglobulins can be noted.

the same test, the difference between the average O.D. value of the two measures and of the control must be at least three times the standard deviation (Figure 1).

Some of the positive clones were analyzed in competition experiments in order to assess if they are indeed antigenic mimics of mAb MM4.17 epitope. Binding of the monoclonal antibody to CEM-VBL100 cells was studied with increasing amounts of phage clones 55, 226, 38, 32, 56c; phage particles displaying wild type pVIII were used as a negative control. The flow cytometry analysis shows that positive phage clones are capable of blocking the staining of P-glycoprotein-expressing cells (Figure 2), demonstrating that all the tested clones compete with the antigen for binding of the mAb MM4.17, while the control phage do not compete, even at the highest concentration. These results indicate that the phage clones interact with the paratope, and thus display positive images of the antigen (i.e. antigenic mimics).

To investigate the structural characteristics peptides capable of mimicking the mAb MM4.17 epitope, the aminoacidic sequences of 26 positive phage clone inserts were obtained through sequencing their encoding DNA, and analyzed according to their sequence similarity. The resulting alignment (obtained using the "Pileup" program of the GCG package (29), Figure 1) surprisingly does not show a unique consensus sequence similar to the previously mapped TRIDDPET linear peptide epitope (14), suggesting that the epitope

	aa #	a	b	c
Ala	21	9.42	6.56	7.66
Arg	14	6.28	9.84	7.97
Asn	6	2.69	3.28	3.01
Asp	14	6.28	3.28	5.26
Cys	2	0.90	3.28	1.37
Gln	4	1.79	3.28	2.80
Glu	17	7.62	3.28	2.68
Gly	8	3.59	6.56	7.15
His	4	1.79	3.28	3.62
Ile	19	8.52	4.92	3.53
Leu	10	4.48	9.84	9.88
Lys	1	0.45	3.28	2.71
Met	20	8.97	1.64	2.10
Phe	6	2.69	3.28	4.41
Pro	33	14.80	6.56	11.43
Ser	17	7.62	9.84	9.30
Thr	6	2.69	6.56	6.20
Trp	2	0.90	1.64	1.89
Tyr	3	1.35	3.28	2.49
Val	16	7.17	6.56	4.56

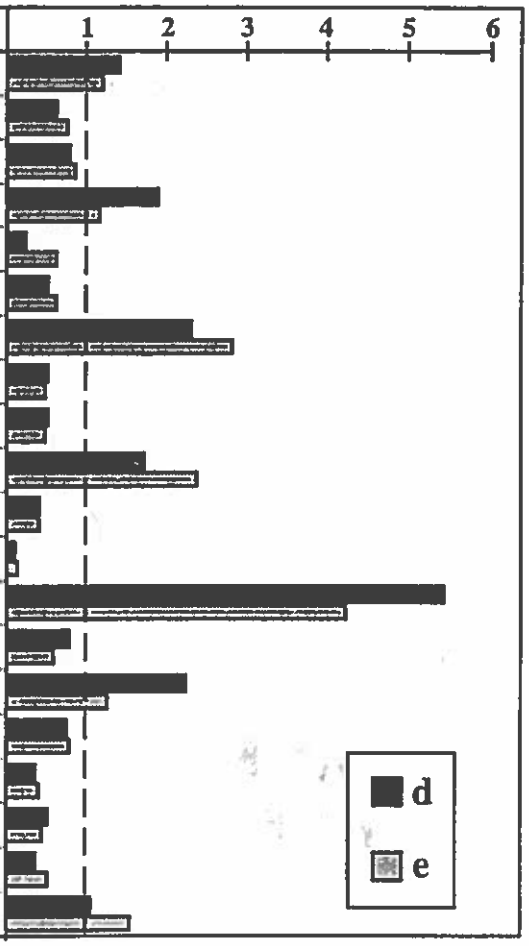


FIGURE 3. Aminoacid composition analysis of the identified positive inserts. aa# indicates the number of times each aminoacid residue occurs in the inserts of positive phage clones. The actual (a), expected (b) and random (c) percentage of each residue in the aminoacid composition are reported (see text). The chart on right panel shows the increase over the expected frequency (indicated by the dashed line) obtained from the ratio a/b or a/c (series d and e, respectively).

of mAb MM4.17 is larger than previously described. Nonetheless, some similarities among the positive sequences could be easily identified; for example, most of the clones contain the dipeptide MI or MV, and the sequence IXP or VXP is almost always present, where the X is often an aspartic acid residue (D). Some of the sequences contain larger regions of similarity, like clones 128 and 129, whose inserts are more than 50% identical.

The overall aminoacid composition of the identified positive inserts was derived and compared with that expected (according to the frequency of the codons in the genetic code, and hence in the degenerated oligonucleotides used to construct the libraries) and with the composition of more than 500 clones selected from the same phage peptide libraries using several different non-related antibodies.

The most striking difference found in the above analysis is a significant enrichment in Methionine (5.5 and 4.3 fold increase over the expected frequency, see Figure 3). Also glutamic acid (2.3 and 2.8) and isoleucine (1.7 and 2.4) were highly enriched; in addition to

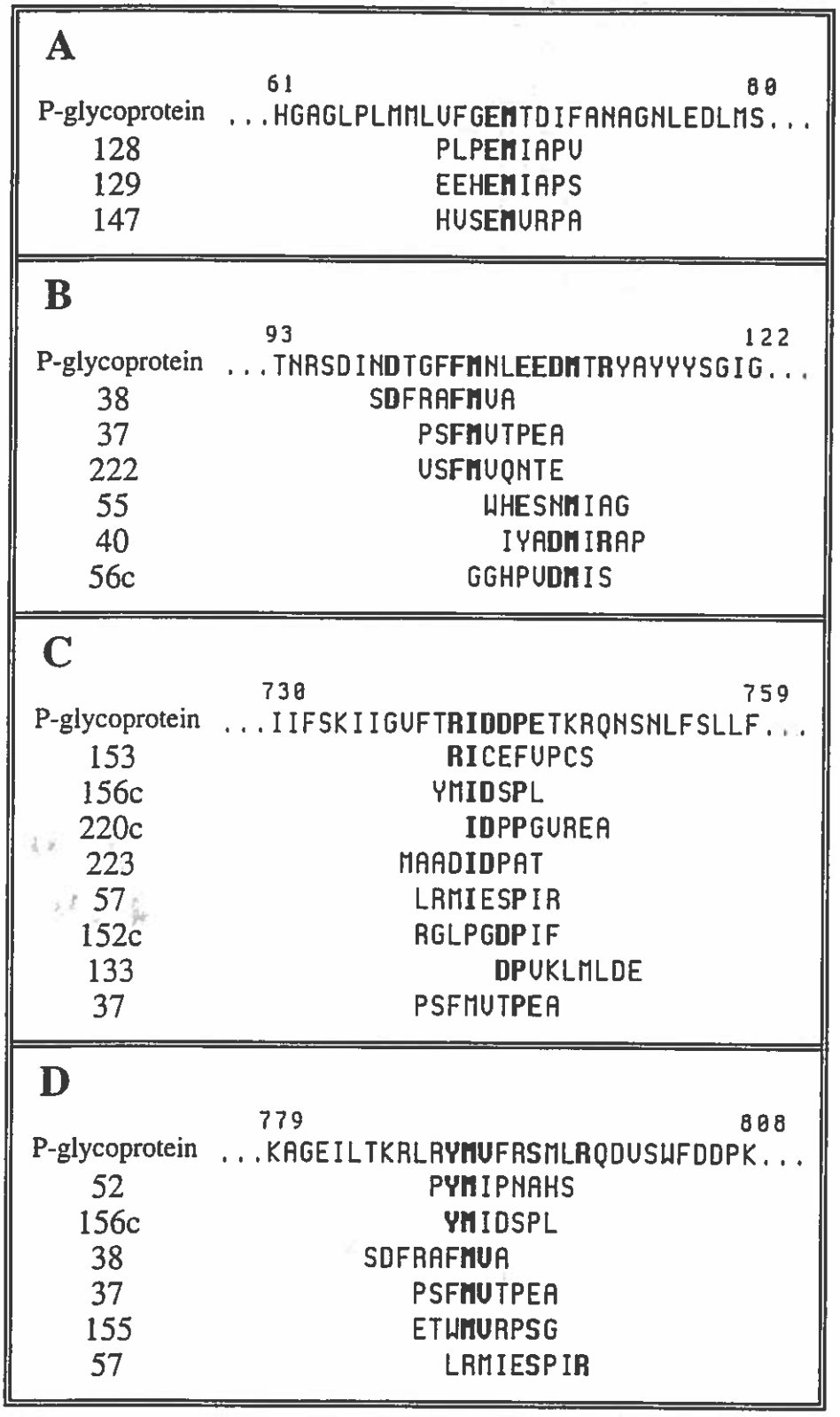


FIGURE 4. Comparison of positive clone sequences with P-glycoprotein. Numbering of P-glycoprotein sequence is according to that in the SWISS-PROT protein database (MDR1-HUMAN, accession number P08183). Insert residues matching the aminoacid sequence of the antigen are indicated in bold.

other aminoacids, such as alanine, aspartic acid, proline and valine, which were also present at a higher frequency than expected.

To ascertain why no positive inserts more similar to the linear epitope sequence TRIDDPET (14) were identified, a phage clone containing the above peptide sequence was constructed. The TRIDDPET-displaying phage reacted very weakly with mAb MM4.17 in ELISA (Figure 1), thus explaining the absence of such clones in the positive population identified through library screening.

The sequences of the positive peptide inserts were compared with that of P-glycoprotein: none of the sequences was detected as clearly corresponding to any region of the primary structure of the antigen. A more elaborate analysis was then performed: all the residues of P-glycoprotein sequence corresponding to the over-represented aminoacid residues were marked, all the most common dipeptide sequences among the positive clones (MI, MV, ID, VD, DP, etc.) were also searched in the antigen sequence, and several possible alignments (obtained using the programs "Fasta" and "Wordsearch" of GCG package (29)) between the sequences of the positive clones and that of P-glycoprotein were analyzed.

From the above procedure, four "clusters" of correspondence along the sequence of P-glycoprotein were identified (Figure 4), indicating four regions of the antigen putatively participating in the epitope structure. One of these four regions (Figure 4, panel C) indeed contains the linear sequence TRIDDPET, previously mapped utilizing synthetic peptides (14), but the epitope is probably larger, including other parts of the molecule; these parts could be represented by all or some of the three other identified regions (Figure 4, panels A, B, D).

It s noteworthy that one of the regions identified as a putative part of the MM4.17 epitope is localized in a segment of the P-glycoprotein molecule postulated to be intracelluar (Figure 4, panel D), similarly to findings in another case of epitope mapping using the same technology (19). In other models recently proposed (20, 21), this segment is suggested as being extracellular. Thus, in the light of antibody epitope mapping data, different possible structures of P-glycoprotein molecules can be hypothesized. Site-directed mutagenesis of the antigen, guided by the similarities found, should allow the precise definition of the mAb MM4.17 epitope, and could corroborate the hypothesis of the existence of alternative structures.

The results deriving from phage peptide library screening with MM4.17 are in accordance with reports on mimicking of non-linear epitopes by peptide sequences (24), where all the positive inserts analyzed appear to contain information (i.e. aminoacid residues) related to more than one region of the antigen primary structure. Taken together, our results suggest that antibody epitopes previously identified as linear through synthetic peptide scanning, could actually be larger than supposed, including also conformational and/or discontinuous antigenic determinants.

The authors thank A. Luzzago and A. Folgori for helpful discussion during this work, P. Neuner for oligonucleotide synthesis, C. Ceracchini for technical help in flow-cytometry analysis and J. Clench for proof-reading of the manuscript. F.P. is deeply grateful to Prof. R. Cortese for the kind hospitality of IRBM.

This work was partly supported by grants from Ministero della Sanità, Istituto Superiore di Sanità (AIDS Research Project).

References

1. Kartner, N., Riordan, J.R. and Ling, V. (1983) *Science 221:*1085-1088.
2. Schinkel, A.H. and Borst, P. (1991) *Semin. Cancer Biol. 2:*213-226.
3. Germann, U.A., Pastan, I. and Gottesman, M.M. (1993) *Semin. Cell Biol. 4:*63-76.
4. Gottesman, M.M., Schoenlein, P.V., Currier, S.J., Brugermann, E.P. and Pastan, I. (1991) In: *Biochemical Aspects of Selected Cancer,* Vol. I, (G. Pretlow and P. Pretlow, eds), Academic Press, San Diego, USA, pp. 339-371.
5. Endicott, J.A. and Ling, V. (1989) *Annu. Rev. Biochem. 58:*137-171.
6. Balzi, E., Chen, W., Ulaszewski, S., Capleaux, E. and Goffeau, A. (1987) *J. Biol. Chem. 262:*16871-16879.
7. Stachel, S.E. and Zambryski, P.C. (1986) *Cell 47:*155-157.
8. Martin, S.K., Oduola, A.M.J. and Milhous, W.K. (1987) *Science 235:*899-901.
9. Ueda, K., Cardarelli, C., Gottesman, M.M. and Pastan, I. (1987) *Proc. Natl. Acad. Sci. 84:*3004-3008.
10. Chen, C.J., Chin, J.E., Ueda, K., Clark, D.P., Pastan, I., Gottesman, M.M. and Roninson, I.B. (1986) *Cell 47:*381-389.
11. Eisenberg, D., Schwarz, E., Komaromy, M. and Wall, R. (1984) *J. Mol. Biol. 179:*125-142.
12. Georges, E., Bradley, G., Gariepy, J. and Ling, V. (1990) *Proc. Natl. Acad. Sci. 87:*152-156.
13. Georges, E., Tsuruo, T. and Ling, V. (1993) *J. Biol. Chem. 268:*1792-1798.
14. Cianfriglia, M., Willingham, M.C., Tombesi, M., Scagliotti, G.V., Frasca, G. and Chersi, A. (1994) *Int. J. Cancer 56:*153-160.
15. Cianfriglia, M., Romagnoli, G., Tombesi, M., Poloni, F., Falasca, G., Di Modugno, F., Castagna, M. and Chersi, A. (1995) *Int. J. Cancer 61:*142-147.
16. Jachez, B., Cianfriglia, M. and Loor, F. (1994) *Anti-Cancer Drugs 5:*655-665.
17. Scott, J.K. and Smith, G.P. (1990) *Science 249:*386-390.
18. Felici, F., Luzzago, A., Monaci, P., Nicosia, A., Sollazzo, M. and Traboni, C. (1995) In: *Biotechnology Annual Review,* (M.R. El-Gewely, ed), Vol. 1, Ch. 5. Elsevier Science B.V., Amsterdam.
19. Poloni, F., Cianfriglia, M. and Felici, F. (1995) *Int. J. Cancer 61:*727-731.
20. Zhang, J.T. and Ling, V. (1991) *J. Biol. Chem. 266:*18824-18232.
21. Skach, W.R., Calayag, M.C. and Lingappa, V.R. (1993) *J. Biol. Chem. 268:*6903-6908.
22. Cenciarelli, C., Currier, S.J., Willingham, M.C., Thiebaut, F., Germann, R., Rutherford, A.V., Gottesman, M.M., Barca, S., Tombesi, M., Morrone, S., Santoni, A., Mariani, M., Ramoni, C., Dupuis, M.L. and Cianfriglia, M. (1991) *Int. J. Cancer 47:*533-543.
23. Felici, F., Castagnoli, L., Musacchio, A., Jappelli, R. and Cesareni, G. (1991) *J. Mol. Biol. 222:*301-310.
24. Luzzago, A., Felici, F., Tramontano, A., Pessi, A. and Cortese, R. (1993) *Gene 221:*1085-1088.
25. Parmley, S.F. and Smith, G.P. (1988) *Gene 73:*305-318.
26. Felici, F., Luzzago, A., Folgori, A. and Cortese, R. (1993) *Gene 128:*21-27.
27. Dente, L., Cesareni, G., Micheli, G., Felici, F., Folgori, A., Luzzago, A., Monaci, P., Nicosia, A. and Delmastro, P. (1994) *Gene 148:*7-13.
28. Sanger, F., Nicklen, S. and Coulson, A.R. (1977) *Proc. Nat. Acad. Sci. 74:*305-318.
29. Program Manual for the Wisconsin Package, Version 8, Genetics Computer Group, Madison, Wisconsin, US, (1994)

Physiol. Chem. Phys. & Med. NMR (1995) 27:281-291

Low Molecular Weight Peptide from Calf's Liver Mitochondrial DNA: Structure and Effect on DNA as a Template

Angela Spena, Rosa Chimenti, Concetta Covello, Tiziana De Cicco, Sergio Mazzulla and Guglielmo Martino

Department of Cell Biology, University of Calabria, I-87030 Rende (CS), Italy

Abstract: A peptide fraction from the mitochondrial DNA of calf's liver was isolated using Drouin's method (1). This peptide fraction, which was extracted at pH 9.5 from an extensively purified mitochondrial DNA (2), has been shown to exert an *in vitro* regulatory role on the transcription and duplication activity of DNA (3). The same fraction also binds with mitochondrial DNA with a high affinity constant and stabilizes DNA from calf's thymus against thermal denaturation. The peptides from mitochondrial DNA have been subfractionated by fingerprinting-like techniques and one of them has been sequenced.

IT IS KNOWN that the DNA of eucaryotic nuclei contains low molecular weight peptides (4, 5). These peptides regulate duplication and transcription in many tissues and in differentiated isolated cells (6, 7). Moreover, studies have demonstrated that the processing of mitochondrial enzyme precursors produces low molecular weight peptides, which are able to regulate nuclear and mitochondrial transcription and replication (8–11) as well as the differentiation in cell cultures (7–12).

The aim of the present study is to demonstrate the presence of low molecular weight peptides bound to mitochondrial DNA and their regulatory capacities in transcriptional and replicative processes. In a subsequent test one of these four peptides, precisely the slowest subfraction in chromatography and the fastest in electrophoresis, has its amino acid composition sequenced.

Materials and Methods

Cell Fractionation

One Kg of wet weight tissue from fresh calf liver was obtained from a local slaughter-house, dissected and external epithelium connective tissue and large vasa were removed. Afterwards the tissue was handled according to Drouin's method (1) as follows: the tissue was washed with a buffer containing 0.25 M Sucrose, 10 mM Tris-HCl pH 7.4, 1 mM EDTA, minced and homogenized in a Potter Helvehjem apparatus at 600 rpm for 6 strokes. The homogenate was then fractionated by differential centrifugation in the same buffer and the isolated mitochondria were lysed with 1.7% SDS in 25 mM Tris-HCl pH 7.5, 50 mM EDTA, 75 mM NaCl buffer. Subsequently extracted mitochondrial DNA was purified on a CsCl gradient (38000 rpm × 36 h in Ti 65 Beckman rotor at 20ºC).

Peptide Isolation

The low molecular weight peptides fraction bound to mitochondrial DNA, was detached by pH jump at pH 9.5 as in Coderoni *et al.* (2). The peptide fraction was dialyzed in Spectra/Por Membrane MWCO 500 against distilled water for 2h and lyophilized.

Spectrophotometric and Spectrometric Characterization of Peptide Fraction

U.V. visible data were obtained in water with a Shimadzu U.V.-2100 spectrophotometer.

I.R. spectra were performed using a Perkin-Elmer 1330 infra-red spectrometer on KBr pellets.

^{31}P NMR spectra were performed using a Bruker 300 MHz spectrometer on deuterium oxide solutions of the peptide fraction. ^{31}P NMR spectra are related to orthophosphoric acid at pH 6.7 (13).

In Vitro Studies on DNA Replication and Transcription

The effects of mitochondrial peptide on DNA replication and transcription have been studied by the spectrofluorometric method of Morgan *et al.* (14, 15).

E. coli DNA polymerase I activity (0.01 U/µl), was determined on Ethidium bromide bound to DNA where d(AT)n-d(AT)n at 0.01 U/µl final concentration was used as template. The reaction mixture contained 15 mM $MgCl_2$, 50 mM K_2HPO_4 pH 7.5, 4 mM d-ATP, 4 mM d-TTP, final concentrations, at 37ºC with a final total volume of 100 µl (16). 5 µl of incubation mixture was added to 2 ml of detection buffer at the indicated times. The detection buffer at pH 8.1 contained 0.5 µg/ml Ethidium bromide (EthBr), 5 mM Tris-HCl pH 8.1, 0.5 mM EDTA, final concentrations, at 25ºC. DNA synthesis was detected in an ISS spectrofluorometer. The excitation wavelength was 525 nm and the emission was 600 nm.

The effect of the peptide fraction was observed at the following final concentrations: 30, 70 and 90 ng/µl.

E. coli RNA polymerase I activity (0.05 U/µl) was studied using *E. coli* DNA at a final concentration of 0.001 U/µl as template both with and without mitochondrial peptides. The reaction mixture contained 40 mM Tris-HCl, 4 mM $MgCl_2$, 1 mM $MnCl_2$, 0.1 mM β-mercaptoethanol, 0.5 mM of each required rNTP, final concentrations, at 37ºC in a total final volume of 100 µl (17). The detection buffer and the study conditions were the same as previously described for DNA polymerase reaction with a final EthBr concentration of 2 µg/ml. The excitation and emission wavelengths were 510 and 600 nm, respectively.

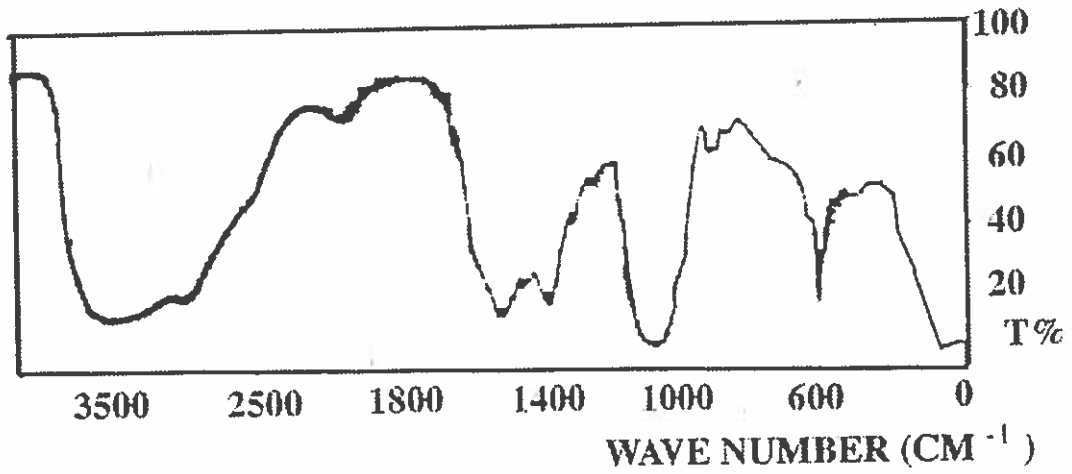


FIGURE 1. I.R. Absorption spectra of mitochondrial purified low molecular weight peptides. Spectra were performed on KBr pellets.

The effect of the peptide fraction was observed at the same final concentrations as previous: 30, 70 and 90 ng/µl.

All highly graded analytical chemicals were purchased from Boehringer Mannheim Germany.

Characterization of Peptide Interactions With DNA

For this purpose the effect of the low molecular weight mitochondrial peptide fraction on the stability of EthBr- DNA calf thymus complexes was evaluated using a fluorometric technique. The observations were performed with an ISS spectrofluorometer and Eth Br binding data were analyzed in accordance with the methods suggested by Le Pecq and Paoletti (18).

The thermal stability of calf thymus DNA was studied with a Shimadzu 2100 UV-VIS spectrophotometer equipped with Temperature Controller SPR-8 melting point apparatus, as in Mandel and Marmur (19), using the following final peptide fraction concentrations: 40, 60, 90 ng/µl, in 0.01 SSC pH 7.

Amino Acids Sequence

The aminoacidic sequence was determined using methods suggested by Chang (20) with DABITC (4-Dimethylaminoazobenzene4′-isothiocyanate) (Fluka) derivatization and bidimensional chromatography on micropolyamide Wang (Schleicher and Schull).

Results and Discussion

The peptide fraction isolated from mitochondrial calf liver DNA was spectroscopically characterized by I.R. and ^{31}P NMR methods.

As described in Figure 1, the fraction has a peptide structure with at least one phosphate group, which binds a hydroxyl group. By I.R. spectroscopy the stretching of N-H bond is evident at 3500 cm^{-1} and at 1650 cm^{-1} the bending of N-H and C=O stretching overlap, at 1170 cm^{-1} the stretching of P=O bond and at 1100 cm^{-1} the stretching of P-O-C bond also

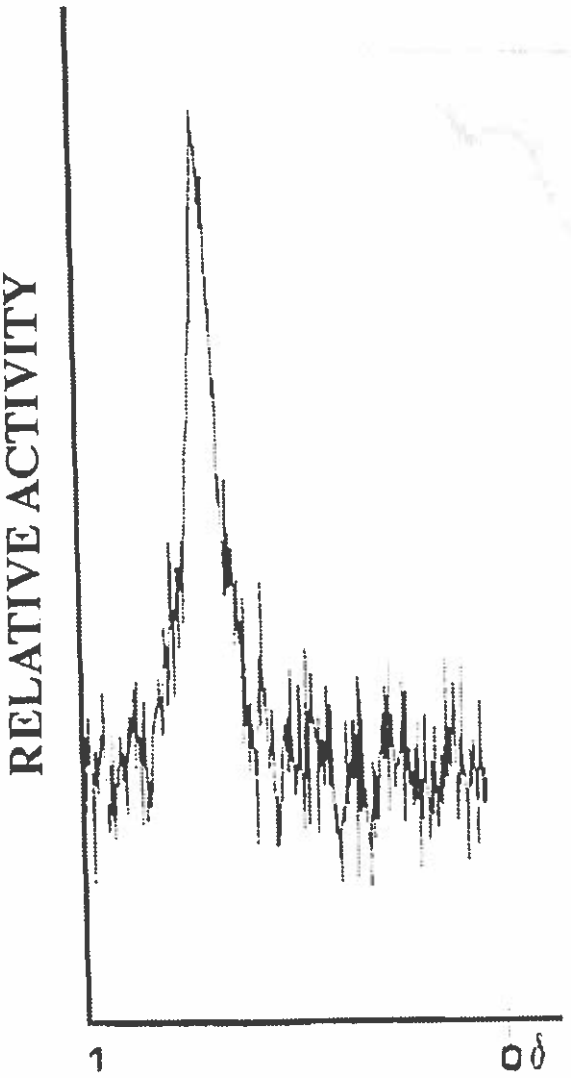


FIGURE 2. ^{31}P NMR spectra of mitochondrial purified low molecular weight peptides. Reference: orthosphosphoric acid pH 6.7 in D_2O.

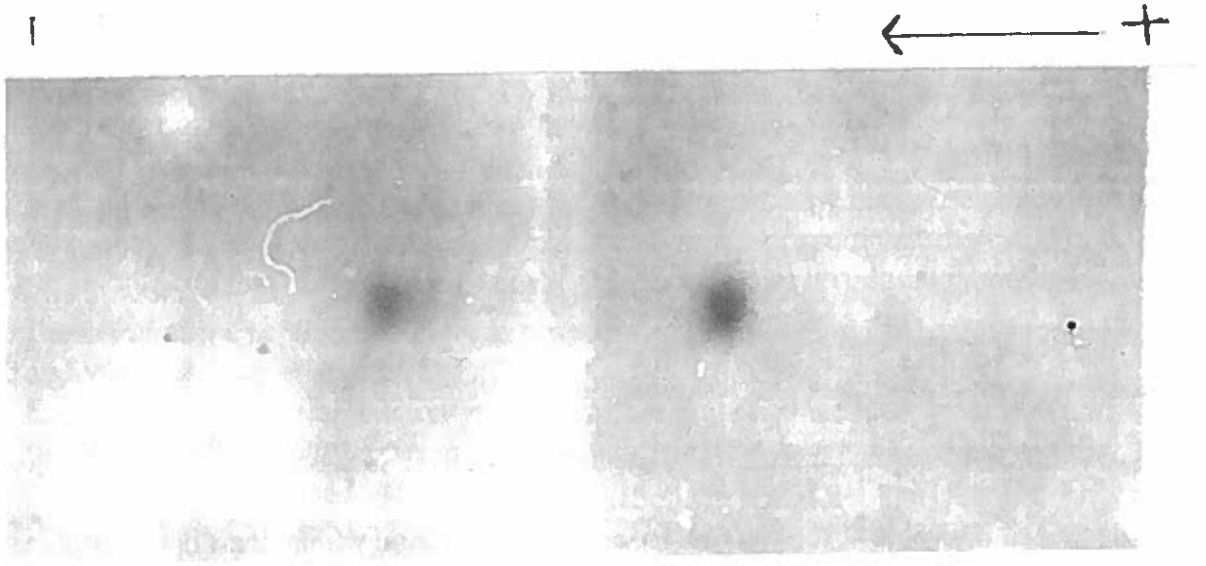


FIGURE 3. Electrophoresis of mitochondrial peptide fraction on 20 × 40 cm sheet Poligram Cel 300. Buffer: 2% Pyridine, 4% Acetic acid, 15% Aceton, 79% Water, pH 4.4. Migration time: 4.5 hrs. 10 mA, 250 V. Staining: 0.4% Ninhydrin in 98% ethanol.

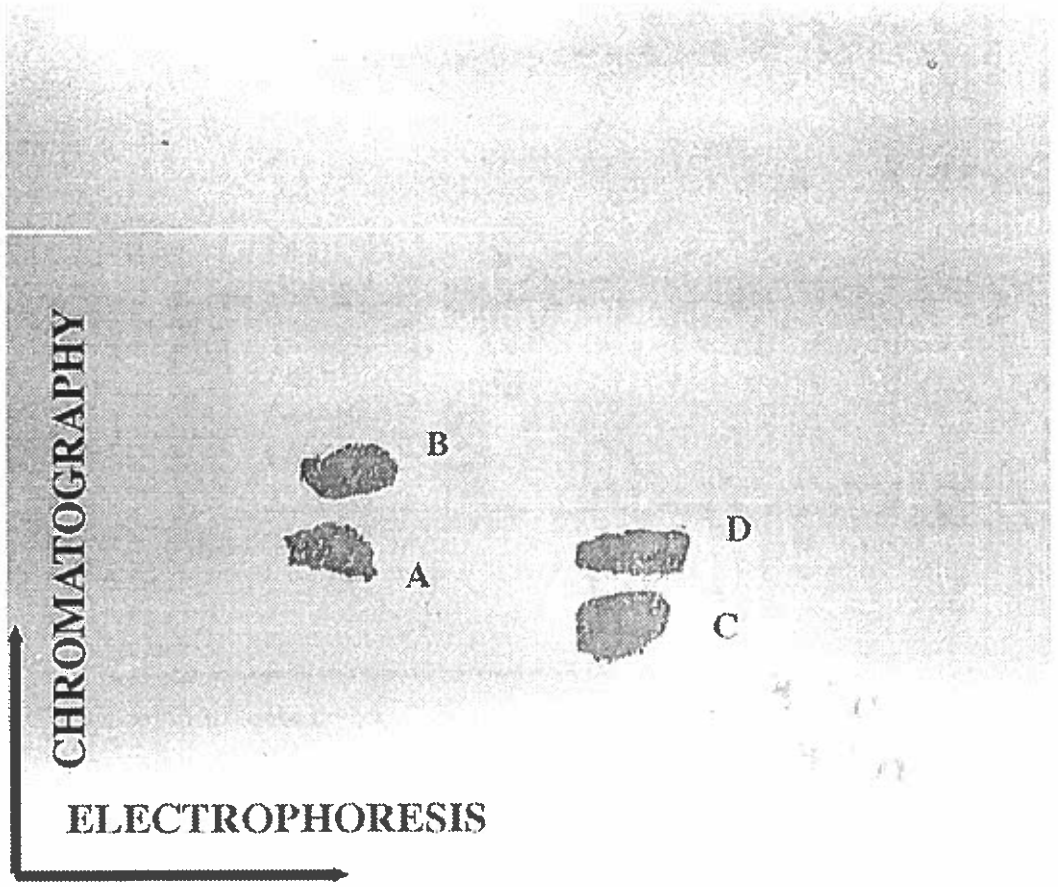


FIGURE 4. Finger-print of mitochondrial peptides on 20 × 20 cm sheet Poligram Cel 300. Electrophoresis: 2% Pyridine, 4% Acetic acid, 15% Acetone, 79% Water, pH 4.4. Migration time: 2 hrs, 10 mA, 250 V. Chromatography: 7.5% Acetic acid, 25% Pyridine, 37.5% n-Buthanol, 30% Water. Migration time: 6 hrs. Staining: 0.002% Fluorescamine in Acetone.

become evident, demonstrating the peptide nature of the sample and the presence of a phosphodiester group.

With ^{31}P NMR the presence of the phosphate group with diester bonds is observable, as evidenced by peak broadening at 0.87 δ (see Figure 2) in the zone of phosphoserine with reference to 85% of H_3PO_4.

The peptide fraction from mitochondrial calf liver DNA seems to be composed of two subfractions with different electrophoretic mobility in 2% pyridine, 4% acetic acid, 15% acetone, 79% water, pH 4.4, at 10 mA, 250 V, on a 20 × 40 cm sheet Poligram Cel 300 (Figure 3). Electrophoresis was performed for 4.5 hrs.

The finger-print of the mitochondrial peptides (Figure 4) has four subfractions by ascendent chromatography in 7.5% acetic acid, 25% pyridine, 37.5% n-butanol, 30% water. The four mitochondrial subfractions are colored red after reaction with ninhydrin. Among the four isolated subfractions only the one slowest in chromatography and the fastest in electrophoresis has been sequenced; here we present its aminoacidic sequence determined by the manual microsequence of Chang (20). The aminoacids sequence of the main chain is mainly composed of the Threo aminoacid, which is most likely modified as in Table I.

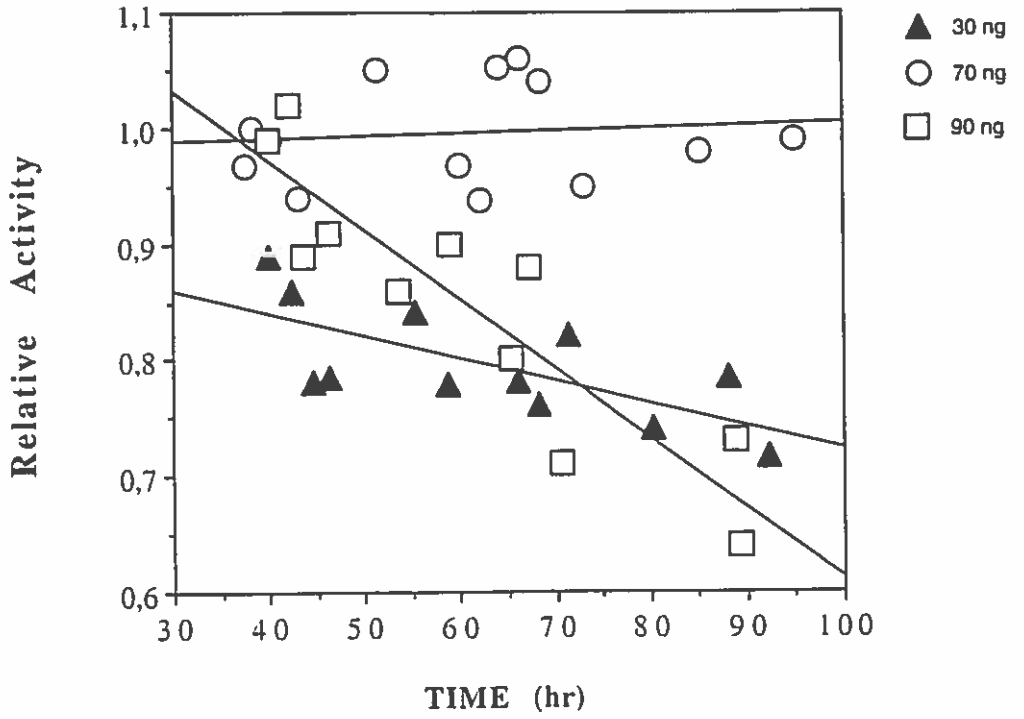


FIGURE 5. Time course of the ratio of DNA synthesis in the presence of mitochondrial peptide against DNA synthesis in the reference condition (16). Enzyme: DNA polymerase I from *E. coli* (0.01 U/µl). Template: d(AT)n-d(AT)n (0.01 U/µl). Detection buffer by Morgan *et al.* (14). Mitochondrial peptide concentration: 30, 70, 90 ng/µl. Each point is the mean of six independent determinations. Regression equations are 30 ng/µl: y = 0.91713 - 1.954e-3X R^2 = 0.495; 70 ng/µl: y = 0.98185 + 2.0638e-4X R^2 = 0.007; 90 ng/µl: y = 1.2111 - 5.9969e-3X R^2 = 0.808.

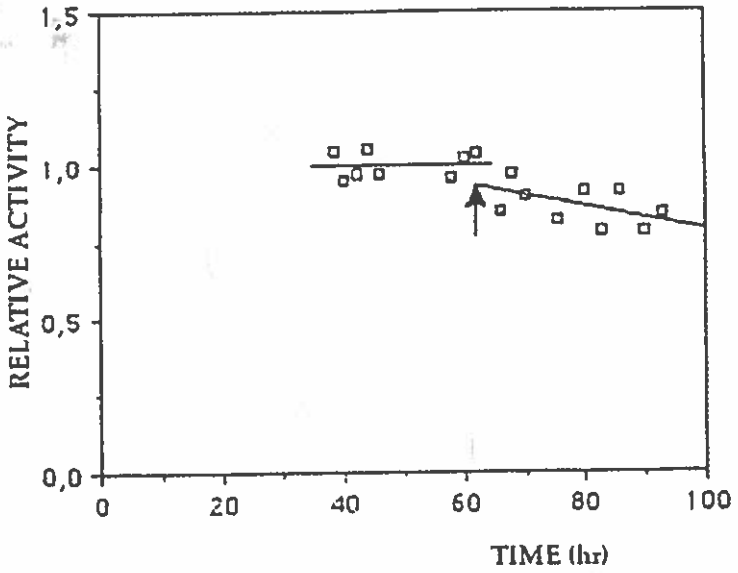


FIGURE 6. Time course of the ratio of DNA synthesis in the presence of mitochondrial peptide against DNA synthesis in the reference condition (16) starting from 62 hrs in the elongation reaction. Enzyme: DNA polymerase I from *E. coli* (0.01 U/µl). Template: d(AT)n-d(AT)n (0.01 U/µl). Detection buffer by Morgan *et al.* (14). Mitochondrial peptide concentration: 50 ng/µl. Each point is the mean of six independent determinations. Regression equations are: up to 62 hrs: y = 0.97087 + 2.8986e-4X R^2 = 0.002; from 62 hrs: y = 1.09050 - 3.6932e-3X R^2 = 0.082.

TABLE I

Aminoacid sequence of peptide fraction C obtained by Chang micro manual method from aminoterminus (20):

Arg
Pro
Threo
Pro
Gly
Threo
Pro
Glu
Met
Threo
Ala

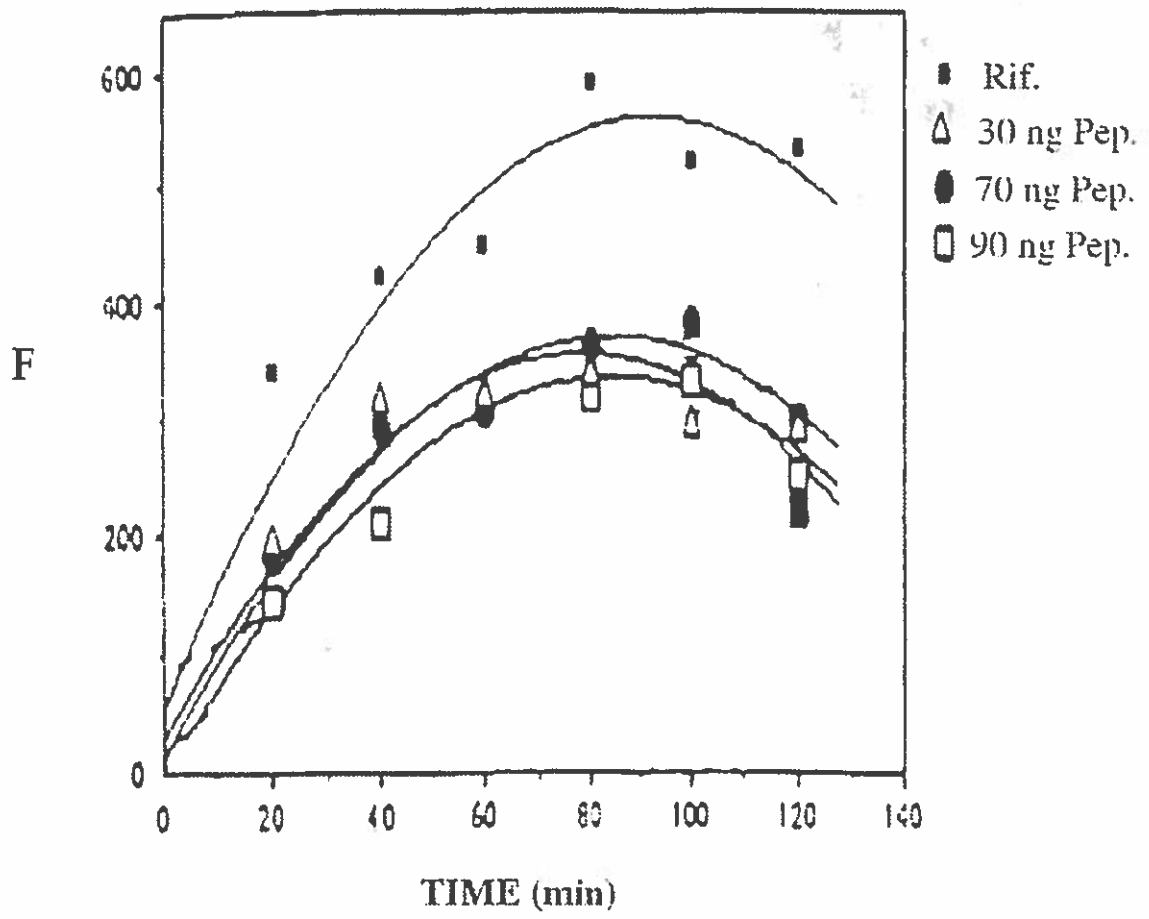


FIGURE 7. Time course of RNA polymerase activity from *E. coli* (type I); concentration 0.05 U/µl (17). Template: DNA E. Coli (0.012 U/µl). Detection buffer by Morgan *et al.* (14). Mitochondrial peptide concentration: 30, 70, 90 ng/µl. Each point is the mean of six independent determinations.

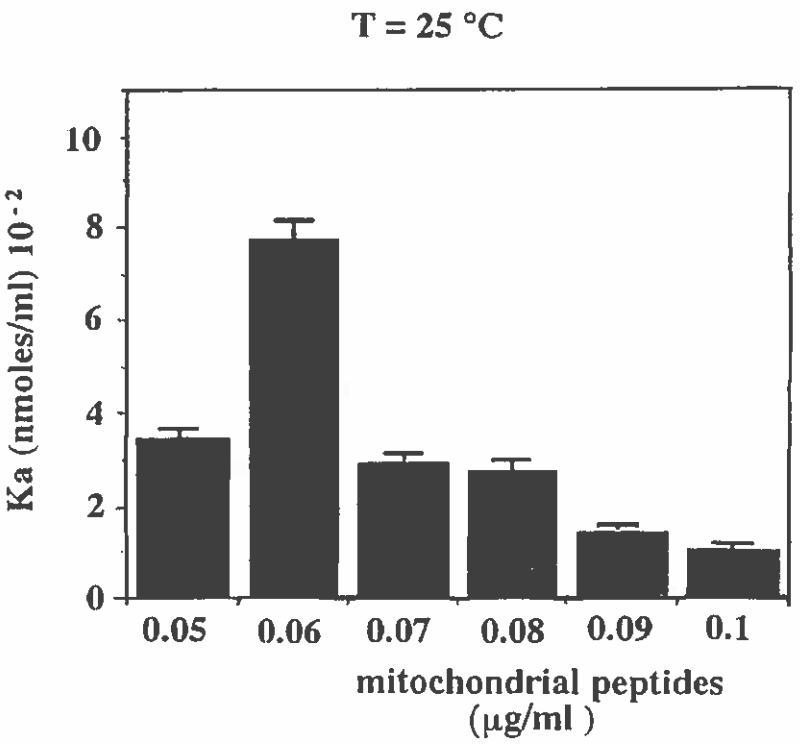


FIGURE 8. Affinity constants of extensively purified calf thymus DNA with EthBr at several mitochondrial peptide concentrations. T= 25ºC. The constants are calculated by means of a Scatchard plot of binding data.

Figure 5 shows the effect of increasing concentrations of peptide added to the incubation mixture on the start-up of the DNA synthesis by *E. coli* DNA polymerase I on poly d(A-T)n-d(A-T)n template.

The spectrofluorimetric results are expressed as relative ratios between observed activities in the presence and in the absence of mitochondrial peptide and show that the synthesis occurs only by a significant amount after 37 hours at 37ºC; the fluorescence decreases remarkably in the presence of peptide after 40 hours of incubation and is always lower than in the reference tests, even with the further addition of enzyme. A negative relationship between relative synthesis activities and time is always observed in all studied concentrations. Furthermore, we observed that the mitochondrial peptide acts upon polymerase activity by inhibition, but that between 30–90 ng/μl the effect is always statistically significant.

Figure 6 shows the effect of mitochondrial peptide on DNA chain elongation with poly d(A-T)n-(A-T)n template in duplication reaction. To see the net effect of mitochondrial peptide on DNA polymerase I, the reaction was observed for 62 hours, and then the peptide was added at a 50 ng/μl final concentration. The spectrofluorometric results are expressed as relative activities between experimental results and data observed in the absense of the mitochondrial peptide. The correlation coefficient of the obtained data is more negative in comparison with the tests of mitochondrial peptide action on duplication initiation. As described in Figure 6 and according to equality tests between the two regressions (21), the effect of this peptide concentration on elongation is significant at a 0.01% level of coincidence.

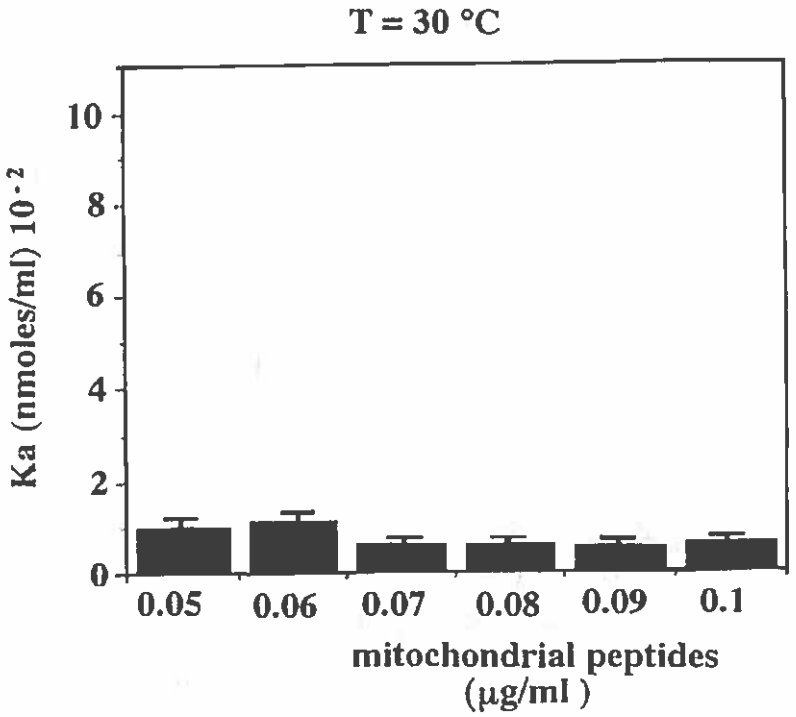


FIGURE 9. Affinity constants of extensively purified calf thymus DNA with EthBr at several mitochondrial peptide concentrations. T = 30ºC. The constants are calculated by means of a Scatchard plot of binding data.

As described in Figure 7, the *E. coli* RNA polymerase I reaction on homologous template is always significantly inhibited by the mitochondrial peptide fraction at all the studied concentrations. This indicates that with circular templates the transcription is always effected by the studied peptides.

The interaction between the extensively purified DNA and Ethidium bromide has been studied at temperatures of 25º, 30º, and 35ºC, at various mitochondrial peptide concentrations by the method indicated in Le Pecq and Paoletti (18). The results show that EthBr affinity, as calculated by Scatchard plot, for purified DNA at pH 9.5 is higher than for nuclear DNA and that, with the increase of the peptide concentration the affinity constant exponentially decreases with purified DNA (Figure 8, 9, 10). In order to further verify the interaction of mitochondrial peptide with nuclear DNA, its action has been studied at concentrations of 40, 60, 90, ng/µl, on the thermal stability of extensively purified calf thymus nuclear DNA. The Tm increases at higher doses of the peptide (Table II).

Conclusions

Results indicate that our mitochondrial peptide fraction has only partial similarity with other kinds of peptides extracted by nuclear DNA sources (22, 23). However, the sequenced fraction evidentiated Threo residues that could be esterified by the phosphate group, as could be seen by I.R. and ^{31}P NMR studies. This structural feature might explain the binding to DNA from several sources, as evidenced by the high affinity with calf thymus DNA and the increase of thermal stability of the same DNA. These data also justify the effects of the

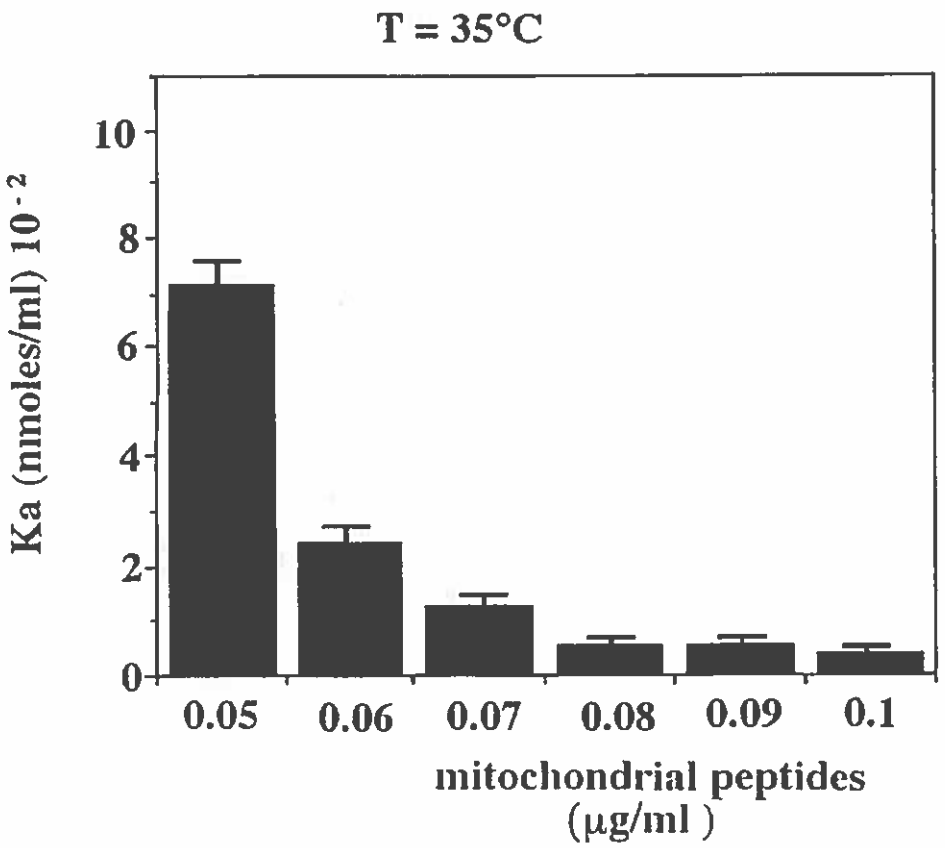


FIGURE 10. Affinity constants of extensively purified calf thymus DNA with EthBr at several mitochondrial peptide concentrations. T = 35ºC. The constants are calculated by means of a Scatchard plot of binding data.

TABLE II

Effect of mitochondrial peptides on T_m of extensively purified calf thymus in 0.1 × SSC

Mitochondrial peptide concentration (ng/µl)	Tm (ºC)
0	63.14* (0.23)
40	62.38* (0.18)
60	63.18* (0.26)
90	64.09* (0.22)

* Each result is the mean of six independent determinations.

mitochondrial peptide fraction on the transcription of *E. coli* DNA and the replication of synthetic templates rich in d(AT)n-d(AT)n.

In summary, even if significant structural differences are evident in this study, the mitochondrial DNA is also shown to bind with low molecular weight peptides probably possessing acidic phosphate groups together with aliphatic backbone and hydroxilic aminoacids. These groups effectively interact with the bases of nucleic acids, simulating the action of peptides from nuclear sources (24).

The hypothesis of Cohen (8, 9) and Blobel (11) that peptides probably arise from the processing of mitochondrial protein precursors is confirmed by our results.

References

1. Drouin, J. (1980) *Med. Biol. 140:*15-34.
2. Coderoni, S., Miano, A., Barra, D., Bramucci, M., Felici, F., Papparelli, M., Amici, D. and Gianfranceschi, G.L. (1988) *Physiol. Chem. Phys. Med. NMR 20:*91-107.
3. Spena, A., Pedace, V., Covello, C., De Cicco, T., Mazzulla, S. and Martino, G. (1993) *J. Biol. Res.-Boll. Soc. It. Biol. Sper. LXIX:*755-759.
4. Gianfranceschi, G.L., Amici, D. and Guglielmi, L. (1976) *Mol. Biol. Rep. 3:*55-64.
5. Guglielmi, L., Gianfranceschi, G.L., Venanzi, F., Polzonetti, A. and Amici, D. (1978) *Mol. Biol. Rep. 4:*195-201.
6. Hillar, M. and Przyjemski, J. (1979) *Biochim Biophys. Acta 564:*246-263.
7. Gianfranceschi, G.L., Hillar, M., Chan, J.T. and Amici, D. (1980) *Mol. Biol. Rep. 6:*95-103.
8. Cohen, P.P. (1981) *Curr. Top. Cell. Regul. 18:*1-19.
9. Cohen, P.P. (1985) *Eur. J. Biochem. 151:*439-444.
10. Bohni, P.C., Daum, G. and Schwartz, G. (1983) *J. Biol. Chem. 258:*4937-4943.
11. Blobel, G. (1980) *Proc. Nat. Acad. 77:*1946-1950.
12. Hillar, M. and Przyjemski, J. (1980) *Mol. Biol. Rep. 6:*67-71.
13. Vogel, H.J. (1989) In: *Methods in Enzymology*, (N.J. Oppenheimer and T.L James, eds), Academic Press, New York, *177:*263-282.
14. Morgan, A.R., Lee, J.S., Pulleyblank, D.E., Murray, N.L. and Evans, D.H. (1979) *Nucl. Acids Res. 7:*547-569.
15. Morgan, A.R., Evans, D.H., Lee, J.S. and Pulleyblank, D.E. (1979) *Nucl. Acids. Res. 7:*571-594.
16. Morgan, A.R., Coulter, M.B., Flintoff, W.F. and Paetkau, V.H. (1974) *Biochemistry 13:*1596-1609.
17. Morgan, A.R. (1970) *J. Mol. Biol. 52:*441-466.
18. Le Pecq, J.B. and Paoletti, C. (1967) *J. Mol. Biol. 27:*87-106.
19. Mandel, M. and Marmur, J. (1968) In: *Methods in Enzymology,* (L. Grossman and K. Moldave, eds), *XII part B:*195-206.
20. Chang, J.Y., Brauer, D. and Wittmann-Liebold, B. (1978) *FEBS Lett. 39:*205-214.
21. Chow, G.C. (1960) "Tests of equality between sets of coefficients in two linear regressions" In: *Econometrica*, Franco Angeli printing house s.r.l., Milano, p. 591.
22. Castigli, E., Mancinelli, L., Mariggiò, M.A. and Gianfranceschi, G.L. (1993) *Am. J. Physiol. 265:*C1220-C1223.
23. Abken, H., Hegger, R., Butzler, C. and Willecke, K. (1993) *Proc. Natl. Acad. Sci. USA 90:*6518-6522.
24. Juodka, B., Phutz, M. and Werner, D. (1991) *Nucl. Acids Res. 19:*6391-6398.

Physiol. Chem. Phys. Med. & NMR (1995) 27:293-301

Analysis of the ATP/GTP Binding Site of Casein Kinase II by Site-directed Mutagenesis

Rolf Jakobi and Jolinda A. Traugh

Department of Biochemistry, University of California, Riverside California

Abstract: Casein kinase II is one of only a few protein kinases which effectively utilize ATP and GTP in the phosphotransferase reaction. Two residues conserved in the ATP binding domain of other protein kinases are unique to the catalytic (α) subunit of casein kinase II. Val-66 is present in subdomain II and Trp-176 in subdomain VII, while > 95% of the other protein kinases contain alanine and phenylalanine, respectively. The residues in the α subunit of casein kinase II were changed to the conserved residues via single and double mutations by site-directed mutagenesis. These mutations enhanced the utilization of ATP over GTP by altering the K_m values of the α subunit for ATP and GTP. Following reconstitution of the catalytic subunit with the regulatory (β) subunit, both the K_m and V_{max} values of the reconstituted $\alpha_2\beta_2$ holoenzyme were altered. Interestingly, the mutations also reduced or eliminated the 4- to 5-fold increase in catalytic activity observed with the holoenzyme over that of the α subunit alone. This was due to changes in secondary structure of the holoenzyme as shown by UV circular dichroism spectroscopy. Taken together, the data indicate that utilization of both ATP and GTP can be directly correlated with stimulation of catalytic activity by the regulatory subunit and suggest a co-evolution of these separate functions.

IN EUKARYOTES, CASEIN KINASE II (CKII) is an ubiquitous multipotential protein kinase, which is second messenger independent and is isolated as an active enzyme. CKII consists of catalytic (α and/or α′) and regulatory (β) subunits which form a physiologically non-dissociable tetrameric structure of $\alpha_2\beta_2$ (Pinna, 1990; Tuazon and Traugh, 1991).

Two characteristics distinguish CKII from most other protein kinases, the effective utilization of ATP and GTP as phosphate donors and the lack of cycling between active and inactive forms. The catalytic and regulatory subunits have been expressed in *E. coli* and the activity of the individual subunits was compared to that of the holoenzyme. These experiments showed that the catalytic subunit alone has a lower catalytic activity than the holoenzyme (Traugh *et al.*, 1990; Hu and Rubin, 1990; Lin *et al.*, 1991; Lin *et al.*, 1992) and that the β subunit stimulates the catalytic activity 4–5 fold (Grankowski *et al.*, 1991; Filhol

et al., 1991; Birnbaum *et al.*, 1992; Jakobi *et al.*, 1992; Lin *et al.*, 1993). Most other protein kinases have regulatory subunits or regulatory domains, which inhibit the catalytic activity, and become activated by dissociation or unfolding of regulatory subunits or regulatory domains in response to external factors.

A comparison of the sequence of CKII with those of other protein kinases (Hanks *et al.*, 1988; Hanks and Quinn, 1991; Benner and Gerloff, 1991) shows that the catalytic subunit of CKII contains two unique amino acids, Val-66 in subdomain II and Trp-176 in subdomain VII, which are conserved in more than 95% of the other protein kinases which contain alanine and phenylalanine in the corresponding positions. Both residues are part of the active site and in close proximity to each other as shown for the cAMP-dependent protein kinase by X-ray crystallographic analysis (Knighton *et al.*, 1991; Taylor *et al.*, 1992; Zheng *et al.*, 1993; Bossemeyer *et al.*, 1993).

Herein, we discuss the effects of single and double mutations of Val-66 and Trp-176 to the conserved residues, Ala-66 and Phe-176, present in the corresponding positions of almost all other protein kinases. Kinetic and structural analyses of wild-type and mutant catalytic subunits and holoenzymes show that utilization of GTP is coupled with the stimulation of catalytic activity by the regulatory subunit and that the latter is caused by a conformational change upon formation of the holoenzyme.

Materials and Methods

Expression of casein kinase II

The α and β subunits of human CKII were cloned individually into pET3a or pT7-7 expression vectors as described by Jakobi and Traugh (1992). The recombinant subunits were expressed in *Escherichia coli* BL21(DE3) following induction with isopropylthiogalactoside (Jakobi and Traugh, 1992). Site-directed mutants of the α subunit were created by the megaprimer PCR method (Sarkar and Sommer, 1990; Jakobi and Traugh, 1992). Recombinant wild-type and mutant subunits of human CKII were purified to apparent homogeneity from bacterial extracts and holoenzymes were reconstituted as described previously (Jakobi and Traugh, 1992; Jakobi and Traugh, 1995).

Assay for casein kinase II activity

CKII activity was determined with dephosphorylated casein and [γ-^{32}P]ATP or [γ-^{32}P]GTP as substrates as described previously (Jakobi and Traugh, 1992) with 100 mM KCl; the activity was measured following precipitation with trichloroacetic acid. K_m and V_{max} values for ATP and GTP were measured by varying the concentration of the labeled nucleotide between 5 and 200 μM for ATP and between 10 and 300 μM for GTP (Jakobi and Traugh, 1992; Jakobi and Traugh, 1995).

Estimation of secondary structure by UV circular dichroism spectroscopy

UV CD spectroscopy was carried out with a JASCO 600 spectropolarimeter between 280 and 180 nm and the secondary structure was estimated by the CONTIN program Version 2 (Provencher and Glöckner, 1981) as described previously (Jakobi and Traugh, 1995).

TABLE I.

Sequence comparison of conserved protein kinase subdomains. Subdomains II and VII of the eleven conserved subdomains of protein kinases are shown for the catalytic subunits of cAMP-dependent protein kinase and casein kinase II. The mutations in the catalytic subunit of CKII are indicated as the substituted amino acids. Amino acids underlined are conserved in almost all protein kinase sequences.

Protein kinase	Subdomain II	Subdomain VII
cAMP-dependent protein kinase	-Tyr-Ala-Met-Lys-Ile-Leu	-Val-Thr-Asp-Phe-Gly
Casein kinase II Wild-type	-Val-Val-Val-Lys-Ile-Leu-	-Leu-Ile-Asp-Trp-Gly
Ala-66	Ala	Trp
Phe-176	Val	Phe
Ala-66/Phe-176	Ala	Phe

Results

Site-directed mutagenesis of amino acid residues unique to the catalytic subunit of casein kinase II

Sequence comparison of conserved protein kinase subdomains (Hanks *et al.*, 1988; Hanks and Quinn, 1991; Benner and Gerloff, 1991) shows that the catalytic α subunit of casein kinase II contains two unique amino acid residues which are conserved in most other protein kinases and are in subdomains involved in nucleotide binding and phosphotransfer. These residues, Val-66 and Trp-176, are present in subdomain II and VII, respectively. More than 95% of all other known protein kinase sequences have alanine and phenylalanine in the corresponding positions. As shown in Table I, these regions are compared to the same regions of the catalytic subunit of cAMP-dependent protein kinase.

Unlike most other protein kinases, CKII utilizes both ATP and GTP effectively as phosphate donors and the regulatory β subunit has been shown to stimulate the catalytic activity of CKII. To examine the functional role of Val-66 and Trp-176 on these unique properties, site-directed mutagenesis of the α subunit was carried out to mutate these residues, singly and in combination, to Ala-66 and Phe-176, the amino acids present in the corresponding positions of most other protein kinases (Table I). Wild-type and mutant α and β subunits of human CKII were expressed in *Escherichia coli* BL21(DE3), and the recombinant proteins were purified to apparent homogeneity and reconstituted to form holoenzymes.

TABLE II.

Comparison of K_m values for ATP and GTP of wild-type and mutant catalytic subunits and reconstituted holoenzymes. At least three different assays were performed for each enzyme and each assay was carried out with duplicate samples. The data shown are calculated average values from these experiments. (Data were compiled from Jakobi and Traugh, 1992, 1995)

	K_m ATP		K_m GTP	
	CKIIα	Reconstituted CKII	CKIIα	Reconstituted CKII
	μM		μM	
Wild-type	13	30	45	47
Phe-176 Mutation	7	21	46	45
Ala-66 Mutation	9	9	71	70
Ala-66/Phe-176 Mutations	6	6	70	72

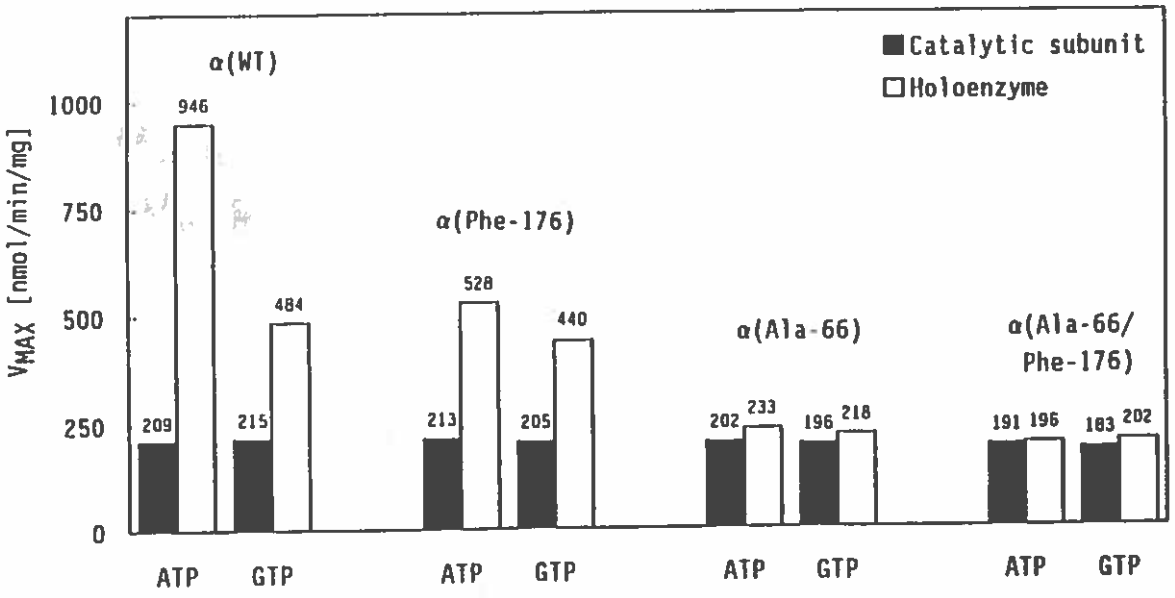


FIGURE 1. Comparison of the V_{max} values for ATP and GTP using catalytic subunits and reconstituted holoenzymes of CKII.

Assays for the wild-type and mutant catalytic subunits and recombinant holoenzymes were carried out with increasing concentrations of ATP and GTP. The V_{max} values are expressed as nmol of phosphate incorporated/min/mg of α subunit. (Data compiled from Jakobi and Traugh, 1992, 1995)

Effects of mutations on phosphotransferase activity

To examine the effects of mutating Val-66 to Ala-66 and Trp-176 to Phe-176, singly and in combination, K_m and V_{max} values for ATP and GTP were determined. The mutations changed the K_m values for ATP and GTP with the purified catalytic subunits alone and with the reconstituted holoenzymes (Table II). The Phe-176 mutation decreased the K_m for ATP from 13 to 7 μM for the catalytic subunit and from 30 to 21 μM for the holoenzyme, while it had no effect on the K_m for GTP for either the catalytic subunit or the holoenzyme. The Ala-66 mutation decreased the K_m for ATP to 9 μM for the catalytic subunit and for the holoenzyme; the K_m for GTP was increased from 45 to 71 μM for the catalytic subunit and from 47 to 70 μM for the holoenzyme. The double mutant of Ala-66/Phe-176 showed the combined effects of both mutations and had the highest affinity for ATP and the lowest affinity for GTP.

For the catalytic subunits alone, the V_{max} values for ATP and GTP were not affected, while for the reconstituted holoenzymes the mutations resulted in a change in V_{max} values for both ATP and GTP (Figure 1). For the catalytic subunits, the V_{max} values for ATP and GTP were all in the same range, about 200 nmol/min/mg. For the holoenzyme, the Phe-176 mutation decreased the V_{max} for ATP from 946 to 528 nmol/min/mg, while the V_{max} for GTP was decreased only slightly from 484 to 440 nmol/min/mg. With the Ala-66 mutation and the double mutant, the V_{max} values for ATP and GTP were decreased to values in the same range as those of the catalytic subunits alone (Figure 1).

The stimulation of V_{max} by the β subunit was 4.5-fold with ATP and 2.3-fold with GTP for the wild-type holoenzyme, when compared to the catalytic subunit alone. With the Phe-176 mutant, this stimulation was reduced to 2.5-fold with ATP but was unaffected with GTP. The stimulation of V_{max} was greatly reduced or eliminated for both ATP and GTP with the Ala-66 and Ala-66/Phe-176 mutations.

Effects of mutations on secondary structure

To examine whether the effects of the mutations on the enzymatic properties were correlated with changes in secondary structure, UV CD spectroscopy was carried out. The CD spectra of wild-type and mutant subunits were examined alone and as the reconstituted holoenzymes and the ratio of α-helices, β-sheets and β-turns/random coils was estimated.

The wild-type and mutant α subunits showed similar spectra and the secondary structure ratios were about 29% α-helices, 38% β-sheets and 33% β-turns/random coils, within the standard errors of estimation (Jakobi and Traugh, 1995). The β subunit showed a very different spectrum, and the secondary structure ratio of 24% α-helices, 59% β-sheets and 17% β-turns/random coils was distinct from that of the α subunits.

The spectra and secondary structure ratios of wild-type and mutant holoenzymes showed significant differences as compared to those of the α subunits (Jakobi and Traugh, 1995). To determine if a conformational change occurred upon holoenzyme formation, the secondary structure ratios of wild-type and reconstituted mutant holoenzymes were compared to the average ratios calculated from the ratios of the corresponding α subunits and the β subunit (Table III). Formation of the wild-type holoenzyme resulted in an increase of α-helices and a decrease in β-sheets. These conformational changes were reduced by the Phe-176 mutation and greatly reduced or eliminated by the Ala-66 and Ala-66/Phe-176 mutations.

TABLE III.

Changes in secondary structure upon reconstitution of wild-type and mutant holoenzymes. Ratios of α-helices, β-sheets and remainder, consisting of β-turns and random coils, were estimated from UV circular dichroism spectra of reconstituted holoenzymes. The data shown were calculated as the difference between reconstituted holoenzymes and the average of the corresponding α and β subunits and therefore represent the changes in secondary structure upon reconstitution of the holoenzymes. (Calculated from data in Jakobi and Traugh, 1995)

Casein Kinase II	Change in		
	α-Helix	β-Sheet	Remainder
Wild-type	+6	-7	+1
Phe-176 Mutation	+4	-1	-3
Ala-66 Mutation	+1	-2	+1
Ala-66/Phe-176 Mutations	+1	-1	0

Discussion

To examine utilization of both ATP and GTP in the phosphotransferase reaction, catalytic subunits and reconstituted holoenzymes with site-specific mutations in the nucleotide binding site were examined and compared with wild-type CKII. With the wild-type holoenzyme, the K_m was 30 μM for ATP and 47 μM for GTP. The catalytic activity of the holoenzyme was 4.5-fold greater with ATP and 2.3-fold with GTP than that of the catalytic subunit alone. This increase was due to an enhanced V_{max}. Mutation of Trp-176 to the consensus amino acid phenylalanine increased the affinity of the catalytic subunit for ATP by decreasing the K_m, but did not affect the K_m for GTP. This mutation reduced the stimulation of catalytic activity observed upon reconstitution to 2.5-fold with ATP by reducing the V_{max}, but had no effect with GTP. Mutation to Ala-66 increased the affinity for ATP and decreased the affinity for GTP by changing the K_m. The double mutant of Ala-66/Phe-176 showed similar effects and had the lowest relative affinity for GTP. Mutation to Ala-66 and Ala-66/Phe-176 greatly reduced or eliminated any effect on the phosphotransferase activity by the β subunit.

Analysis of the catalytic subunit by UV CD spectroscopy showed no significant effect of the mutations on the secondary structure (Jakobi and Traugh, 1995). Reconstitution of the wild-type holoenzyme resulted in an increase of α-helices and a decrease of β-sheets when compared to the average values for the individual subunits, and confirms previous studies by Issinger *et al.* (1992). For the Phe-176 holoenzyme, the increase of α-helices was diminished as compared with the wild-type holoenzyme and was coincident with a decrease in β-turns/random coils. The secondary structures of the Ala-66 and Ala-66/Phe-176 holoenzymes were the same as the combined average values of the individual subunits; no changes in secondary structure were observed upon holoenzyme formation.

Thus, the degree of conformational change observed upon formation of the holoenzyme is strictly correlated with the degree of stimulation of catalytic activity by the β subunit. The change in secondary structure appears to be the molecular event responsible for the en-

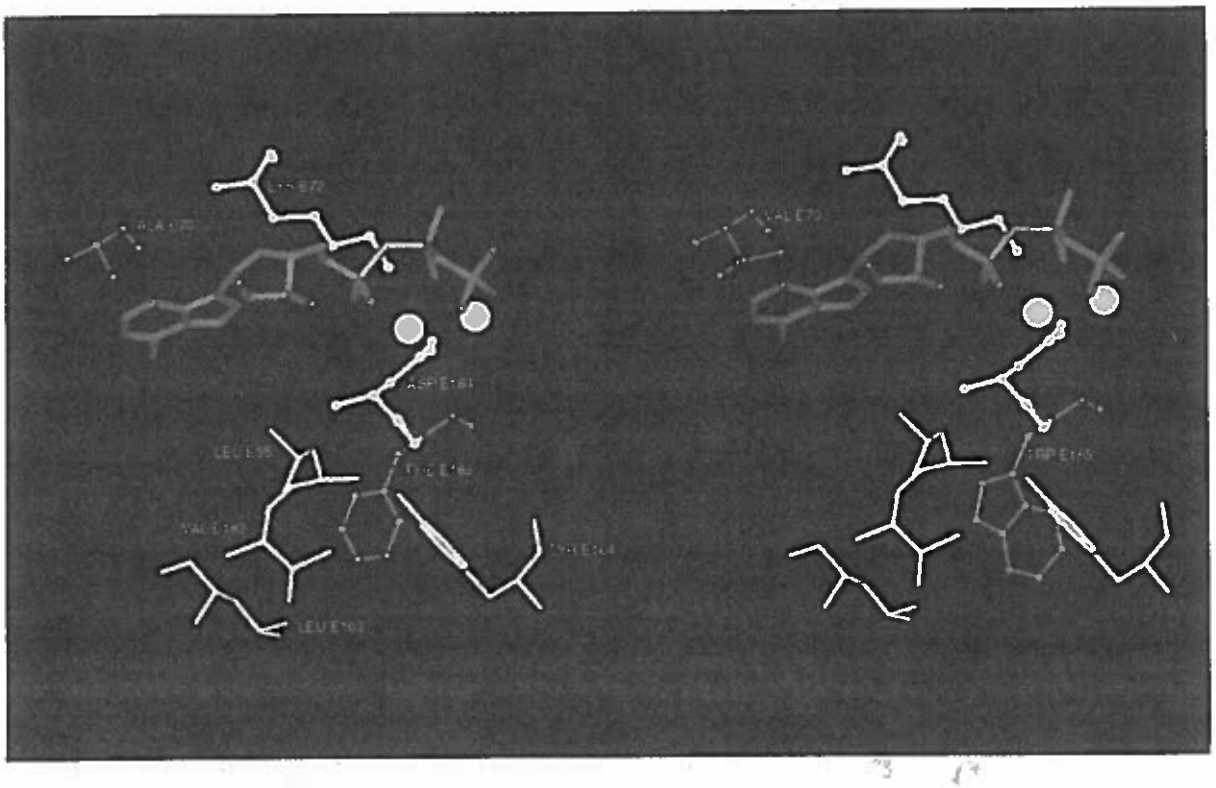


FIGURE 2. Specific amino acid residues in the nucleotide binding domain.

Left Panel, specific amino acid residues in the ATP binding domain of the catalytic subunit of the cAMP-dependent protein kinase are identified. ATP is shown in broad grey lines, two manganese ions as solid circles. Right Panel, the conserved residues Ala-70 and Phe-185 have been replaced with valine and tryptophan, respectively, the amino acid residues in the corresponding positions of CKII. Modified from the Brookhaven Protein Data Bank entry 1ATP (Zheng *et al.*, 1993).

hanced catalytic activity and can be directly correlated with the presence of residues Val-66 and Trp-176. Since the same residues are also involved in the effective utilization of GTP, these unique properties of CKII are probably the result of co-evolution.

Due to the high level of sequence conservation in the family of protein kinases, the X-ray crystallographic structure of the catalytic subunit of cAMP-dependent protein kinase can serve as a structural framework for the protein kinase family (Knighton *et al.*, 1991; Taylor *et al.*, 1992; Zheng *et al.*, 1993; Bossemeyer *et al.*, 1993). Comparison of this structure with those of cell cycle dependent protein kinase 2 (Cdk2) and mitogen-activated protein kinase (MAPK/ERK2) showed a high degree of structural conservation of the catalytic core (Taylor and Radzio-Andzelm, 1994).

In the X-ray crystallographic structure of the catalytic subunit of cAMP-dependent protein kinase, residues corresponding to Val-66 and Trp-176 in CKII, Ala-70 and Phe-185, respectively, are associated with the nucleotide binding site (Figure 2, Left Panel). Ala-70 is part of a β-sheet and located two residues N-terminal to the invariant lysine which contacts the α and β phosphates of the ATP (shown in broad grey lines). The side chain of Ala-70 is close to the purine ring of the nucleotide. A valine residue in this position, as in the α subunit of CKII, could interact differently with the purine rings of ATP and GTP (Figure 2, Right Panel). This could result in the differential utilization of ATP and GTP

observed between the wild-type and the Ala-66 mutant. This mutation did not result in a change of the secondary structure of the catalytic subunit as shown by UV CD spectroscopy, but resulted in a diminution of stimulation by the β subunit.

Phe-185 is in the middle of two conserved residues, aspartic acid which chelates Mg^{2+} bound to the β and γ phosphates of the nucleotide and glycine. The phenyl group of Phe-185 is part of a hydrophobic pocket with Leu-95, Leu-103, Tyr-164 and Val-182. The function of this hydrophobic pocket is to bring functional residues like Asp-184 into appropriate orientations (Figure 2, Left panel). A tryptophan residue in the corresponding position of the α subunit of CKII could loosen the hydrophobic pocket (Figure 2, Right Panel). This could alter the interactions between Asp-184, other amino acid residues and the nucleotide, which would explain the enhanced utilization of ATP observed with the Phe-176 mutant. This mutation did not result in a change of the secondary structure of the catalytic subunit as shown by UV CD spectroscopy, but reduced the enhanced catalytic activity observed with the holoenzyme.

If the K_m values for ATP and GTP also reflect affinities for ADP and GDP, a change in the binding affinity of ATP or GTP would have the reverse effect on the release of ADP and GDP. The easiest way to interpret the effects of the Ala-66 and Phe-176 mutations would be to assume that the rate-limiting step of V_{max} with the catalytic subunits is the turn-over rate, while for the holoenzymes it is the release of products. For the catalytic subunits, a mutation which alters the K_m for ATP and/or GTP would only affect binding, while for the holoenzymes it would also change the release rate. The different V_{max} values for ATP and GTP of the wild-type and mutant holoenzymes would suggest that the β subunit stimulates the turn-over rate with ATP and GTP, and stimulates the release rate with ATP, but not with GTP. According to this interpretation, the Phe-176 mutation would reduce stimulation of the release of ADP but would not affect the stimulation of the turn-over rate. The Ala-66 mutation would eliminate stimulation of V_{max} of the β subunit by reducing stimulation of the turn-over and the release of ADP and GDP.

This research was supported by a grant from the United States Public Health Service GM26738. Dr. Rolf Jakobi was supported by a research scholarship (Ja 557/1-1) from the Deutsche Forschungsgemeinschaft. We wish to thank Drs. Sarah Cox and Susan S. Taylor, University of California, San Diego, for helpful discussions and assistance in locating Val-66 and Trp-176 in the crystal structure of the catalytic subunit of cAMP-dependent protein kinase, and Ms. Marie Green of the Center for Visual Computing, University of California, Riverside, for computing and scientific visualization.

References

Benner, S.A. and Gerloff, D. (1991) *Adv. Enzyme Reg. 31:*121-181.

Birnbaum, M.J., Wu, J., O'Reilly, D.R., Rivera-Marrero, C.A., Hanna, D.E., Miller, L.K. and Glover, C.V.C. (1992) *Protein Expression and Purification 3:*142-150.

Bossemeyer, D., Engh, R.A., Kinzel, V., Ponstingl, H. and Huber, R. (1993) *EMBO J. 12:*849-859.

Filhol, O., Cochet, C., Wedegaertner, P., Gill, G.N. and Chambaz, E.M. (1991) *Biochemistry 30:*11133-11140.

Grankowski, N., Boldyreff, B. and Issinger, O.-G. (1991) *Eur. J. Biochem. 198:*25-30.

Hanks, S.K., Quinn, A.M. and Hunter, T. (1988) *Science 241:*42-52.

Hanks, S.K. and Quinn, A.M. (1991) *Methods Enzymol. 200:*38-62.

Hu, E. and Rubin, C.S. (1990) *J. Biol. Chem. 265:*20609-20615.

Issinger, O.G., Brockel, C., Boldyreff, B. and Pelton, J.T. (1992) *Biochemistry 31:*6098–6103.
Jakobi, R. and Traugh, J.A. (1992) *J. Biol. Chem. 267:*23894–23902.
Jakobi, R., and Traugh, J.A. (1995) *Eur. J. Biochem. 230:*1111–1117.
Knighton, D.R., Zheng, J., Ten Eyck, L.F., Ashford, V.A., Xuong, N.-H., Taylor, S.S. and Sowadski, J.M. (1991) *Science 253:*407–414.
Lin, W.-J., Tuazon, P.T. and Traugh, J.A. (1991) *J. Biol. Chem. 266:*5664–5669.
Lin, W.-J., Palen, E., Tuazon, P.T. and Traugh, J.A. (1992) In: *Recent Advances in Cellular and Molecular Biology,* (R.J. Wegmann and M.A. Wegmann, eds), Vol. 4, pp. 29–34.
Lin, W.-J. and Traugh, J.A. (1993) *Protein Expression and Purification 4:*256–264.
Pinna, L.A. (1990) *Biochim. Biophys. Acta 1054:*267–284.
Provencher, S.W. and Glöckner, J. (1981) *Biochemistry 20:*33–37.
Sarker, G. and Sommer, S.S. (1990) *BioTechniques 8:*404–407.
Taylor, S.S., Knighton, D.R., Zheng, J., Ten Eyck, L.F. and Sowadski, J.M. (1992) *Annu. Rev. Cell Bio. 8:*429–462.
Taylor, S.S. and Radzio-Andzelm, E. (1994) *Structure 2:*345–355.
Traugh, J.A., Lin, W.-J., Takada-Axelrod, F. and Tuazon, P.T. (1990) In: *The Biology and Medicine of Signal Transduction* (Y. Nishizuka, M. Endo and C. Tanaka, eds), Raven Press, New York, pp. 224–229.
Tuazon, P.T. and Traugh, J.A. (1991) In: *Advances in Second Messenger and Phosphoprotein Research* (P. Greengard and G.A. Robison, eds), Vol. 23, Raven Press, New York, pp. 123–164.
Zheng, J., Knighton, D.R., Ten Eyck, L.F., Karlsson, R., Xuong, N.-H., Taylor, S.S. and Sowadski, J.M (1993) *Biochemistry 32:*2154–2161.

Physiol. Chem. Phys. & Med. NMR (1995) 27:303-311

Functional Domains of Nucleolar Phosphoprotein p120

Egon Durban[1], Benigno C. Valdez[1], William C. Gustafson[1], Charles W. Taylor[1], Elena Cardellini[2] and Harris Busch[1]

[1]Baylor College of Medicine, Department of Pharmacology, Houston, Texas 77030 and
[2]Department of Cellular and Molecular Biology, University of Perugia, Perugia, Italy

Abstract: Nucleolar phosphoprotein p120 is a low abundance, proliferation-associated protein. Several functional domains have been characterized and are discussed here such as the antigenic domain recognized by a monoclonal antibody, the nuclear/nucleolar localization domain, phosphorylation domains of casein kinase II (CKII) and protein kinase C, a putative methylation domain and an RNA binding region. By sucrose gradient sedimentation analyses, protein p120 was shown to rapidly sediment with 60-80 S pre-rRNP particles but sedimented more slowly when treated with RNAse or salt suggesting binding to RNA. Nucleolar protein p120 differed from other nucleolar proteins such as C23 (nucleolin) and B23 (nucleophosmin) which sedimented more slowly near the top of the gradient.

PHOSPHOPROTEIN P120 is a low-abundance, proliferation-associated nucleolar protein which was originally identified by monoclonal antibodies (Freeman *et al.*, 1988). Evidence for a role of p120 in cell proliferation was obtained by microinjection of anti-p120 monoclonal antibodies into HeLa cells: cell growth and DNA and RNA synthesis were inhibited (Freeman and Bondada, 1990). Furthermore, growth of NIH/3T3 cells was increased by transfection with p120 cDNA and growth was inhibited by a p120 antisense construct (Perlaky *et al.*, 1992).

The full amino acid sequence was derived from a cDNA clone (Fonagy *et al.*, 1989) and the genome structure including upstream control regions have been characterized (Larson *et al.*, 1990). Availability of the p120 cDNA provided the basis for the studies on functional regions discussed in this paper.

Materials and Methods

Nucleolar Extracts. Nucleoli were isolated from 10 g HeLa cells as described previously (Muramatsu *et al.*, 1974). Nucleolar pellets were extracted with 10 mM Tris-HCl, pH 8.0, 0.15% deoxycholate, 10 mM KCl and 0.5 mM $MgCl_2$. Phenylmethylsulfonyl fluoride (1 mM), leupeptin (1 ug/ml) and Aprotinin (1 ug/ml) were added to the extraction solution as protease inhibitors.

Sucrose density gradient ultracentrifugation. Nucleolar extracts were carefully layered on 5–45% sucrose gradients and centrifuged at 100,000 g for 17 hr in a SW41 rotor. Fractions of 0.9 ml were collected and analyzed by ELISA, Western blot, or RNA gel electrophoresis.

ELISA assays. ELISA assays were performed as described previously (Valdez *et al.*, 1990a). Aliquots (50 ul) of the sucrose gradient fractions were bound to IMMULON-2 microtiter plates (Dynatech Laboratories, Inc., Alexandria, VA) overnight at 4ºC. Wells were then blocked with 3% bovine serum albumin/10% goat serum, incubated with monoclonal p120 antibodies, goat anti-mouse IgG coupled to peroxidase, and finally substrate (2,2′-azino-di-3-ethylbenzthiozolinesulfonic acid, Boehringer Mannheim) color development was measured by absorbance at 405 nm.

Western immunoblots. Sucrose-gradient fractions were concentrated by precipitation with trichloroacetic acid (10%). Pelleted proteins were electrophoresed on 7.5% acrylamide-gels containing 0.1% SDS (Laemmli, 1970). Proteins were transferred to nitrocellulose (Towbin *et al.*, 1979), blocked and then treated with anti-p120 monoclonal antibody followed by alkaline phosphatase-conjugated goat-anti-mouse IgG. Color development was with 5-bromo-4-chloro-3-indoyl phosphate (BCIP) and nitro-blue tetrazolium (NBT) substrate dyes (Promega).

RNA electrophoresis. RNA was isolated from two-fraction pools by phenol-chloroform extraction and ethanol precipitation and was electrophoresed in 1% agarose gels containing 3% formaldehyde (Sambrook *et al.*, 1989).

Phosphorylation of p120 with protein kinase C and phosphopeptide analysis. Protein kinase C was purified from rat brain as described (Cardellini and Durban, 1993). Recombinant protein p120 expressed in *E. coli* was gel-purified and phosphorylated with protein kinase C under the conditions described (Cardelllini and Durban, 1993). Phosphorylated p120 was subjected to tryptic digestion followed by two-dimensional electrophoresis/chromatography (Cardellini and Durban, 1993; Boyle *et al.*, 1991).

Results and Discussion

Protein p120 Antigenic Domain. The first functional domain of p120 to be identified was the antigenic domain that interacts with the monoclonal antibody. A series of deletion mutants of the p120 cDNA were expressed in *E. coli* and probed with monoclonal antibody on immunoblots (Valdez *et al.*, 1990a). Based on these studies and immune competition assays with synthetic peptides, the epitope of the anti-p120 monoclonal antibody was

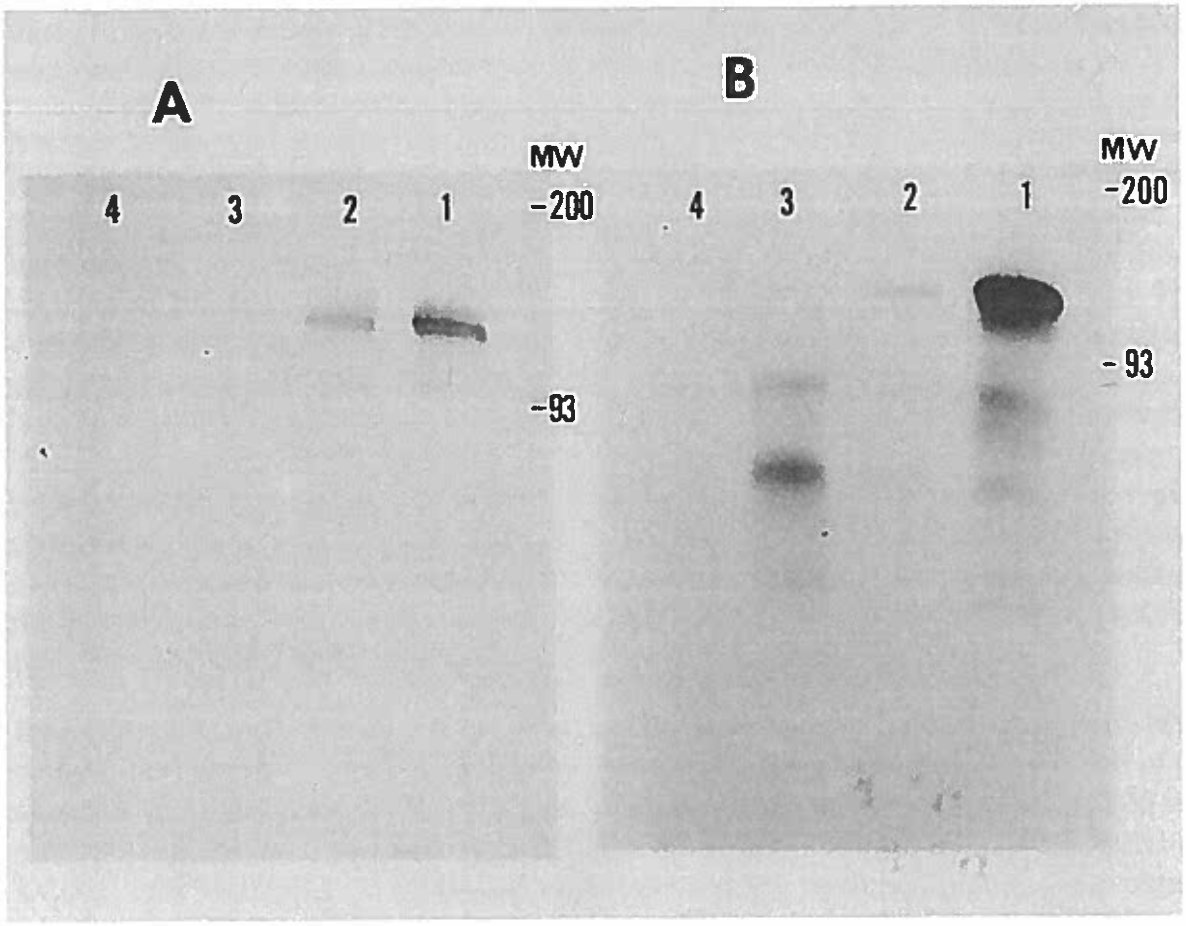


FIGURE 1. Phosphorylation of protein p120 with protein kinase C. Gel-purified recombinant protein p120 was phosphorylated with protein kinase C at 30ºC and electrophoresed on a 7.5% SDS/polyacrylamide gel. A) Western blot using anti-p120 monoclonal antibody; B) Autoradiogram. Lane 1: the phosphorylation assay contained 1 ug of protein p120, 5 mM $MgCl_2$, phosphatidylserine (40 ug/ml), diolein (8 ug/ml), 12.5 uCi [γ-^{32}P]ATP (4.2 uM final concentration), 0.5 mM $CaCl_2$ and 0.04 units of protein kiase C in 100 ul total volume. The reaction was stopped with 100 ul Laemmli sample buffer (Laemmli, 1970). Lane 2: as lane 1, but replacing phosphatidylserine, diolein and $CaCl_2$ with 1 mM EGTA. Lanes 3 and 4: as lanes 1 and 2, respectively but without p120.

localized at amino acid residues 173–180 (EAAAGIQW) (Valdez *et al.*, 1990a). The antibody-antigen interaction was shown to be highly specific inasmuch as (i) a synthetic peptide comprising the epitope region did not compete with binding of anti-B23 monoclonal antibody to its antigen, nucleolar protein B23; (ii) in total HeLa cell extract, only one single band at 120 kDa reacted with p120 monoclonal antibody; and (iii) the antibody did not cross-react with p120 homologues of other species (e.g. mouse, Sf 9 insect cells, CHO cells). For mouse p120, the sequence corresponding to the epitope in human p120 was shown to deviate significantly (DATTGVLW in mouse versus EAAAGIQW in human) (Valdez *et al.*, 1992).

Phosphorylation Domains. *In vivo* labeling of HeLa cells with P32-orthophosphate showed that p120 was a phosphoprotein phosphorylated at ser-, thr-, and tyr-residues (Valdez *et al.*, 1990b). A tryptic peptide containing the epitope domain and an acidic cluster

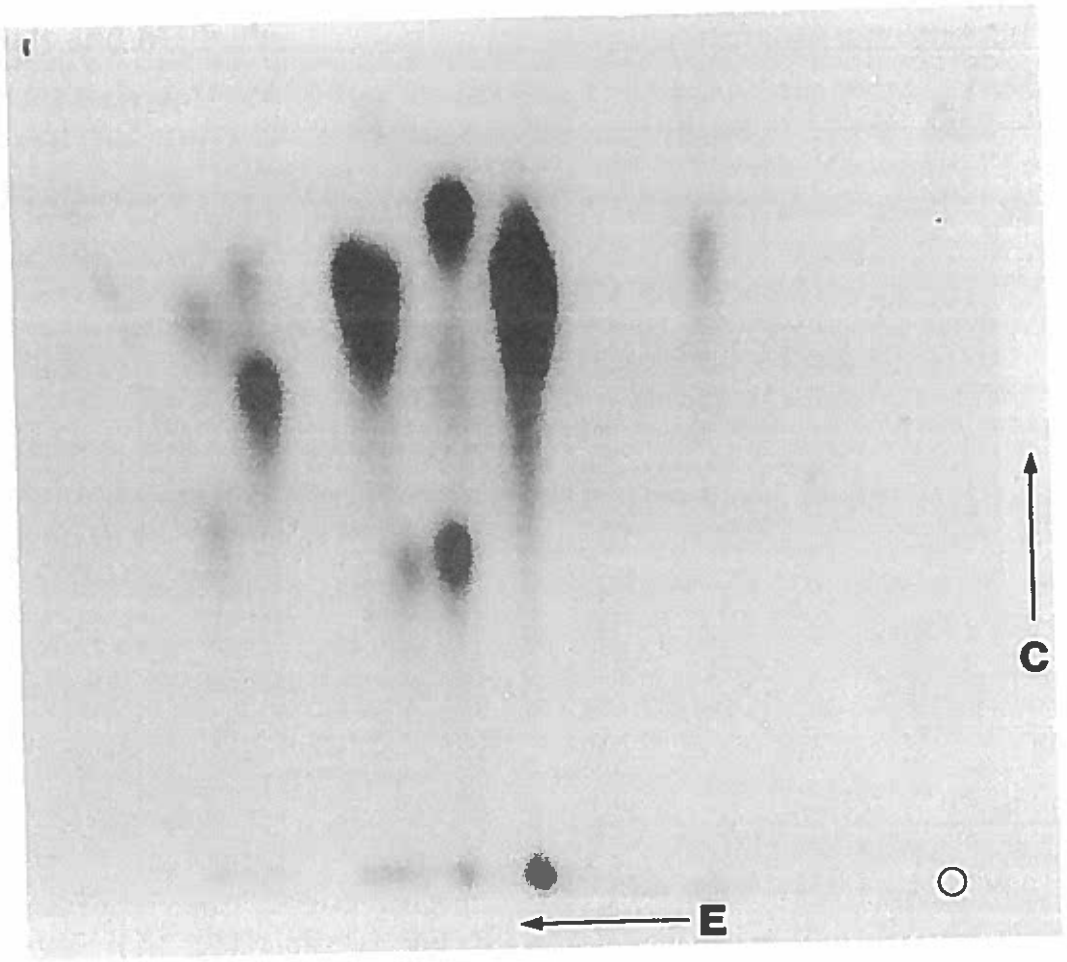


FIGURE 2.. Two-dimensional map of p120 tryptic phosphopeptides. Protein p120 was phosphory lated with protein kinase C as described in Figure 1. The phosphorylated p120 band was excise from the SDS gel, digested with trypsin, loaded on thin layer cellulose plates and subjected to higl voltage electrophoresis in acetic acid/formic acid/water (15:5:80) (first dimension) followed by as cending chromatography in n-butanol/pyridine/acetic acid/water (32.5/5/25/20) (second dimension) O, origin; E, electrophoresis from positive to negative pole; C, chromatography.

C-terminal to the epitope domain (E[173]AAAGIQWS[181]EEET[185]EDEEEEK[192] was expressed as a fusion protein with the maltose-binding protein (Duplay *et al.*, 1987) purified and phosphorylated with purified CKII kinase. Upon digestion with trypsin fol lowed by two-dimensional electrophoresis/chromatography on thin layer cellulose (TLC plates (Boyle *et al.*, 1991), one tryptic phosphopeptide was detectable which comigrate with a phosphopeptide labeled *in vivo*; this suggested that casein II kinase phosphorylate 120 at ser-181 and thr-185 in the intact cell (Valdez *et al.*, 1990b).

In addition to potential CKII sites, p120 contains several potential protein kinase phosphorylation sites. To test this experimentally, recombinant p120 purified from *E. co* extracts was incubated with protein kinase C purified from rat brain as described (Cardelli and Durban, 1993). As shown in Figure 1, p120 was phosphorylated in a phospholipid-d pendent manner. The p120 band was excised, digested with trypsin and subjected 2-dimensional peptide analysis. A number of labeled phosphopeptides were detectab indicating that virtually all of the 9 protein kinase C-sites predicted by the Eugene comput program (Valdez *et al.*, 1992; Lawrence *et al.*, 1989), were actually phosphorylated

protein kinase C *in vitro* (Figure 2). However, the number of sites phosphorylated *in vivo* is likely to be much smaller as they may not be accessible due to p120's *in vivo* interactions with other proteins or RNA.

Nuclear/nucleolar localization signal. A systematic series of p120 cDNA deletion mutants were placed in front of the *E. coli* lacZ gene, transfected into NIH 3T3 cells and expressed as polypeptide-fusion proteins; location of β-galactosidase was monitored by immunofluorescence using anti-β-galactosidase antibodies (Valdez *et al.*, 1994). The fusion protein containing p120 residues 99–110 (NAPRGKKRPAPG) was localized in nuclei. This sequence is homologous to the reported consensus sequence for the monopartite nuclear localization signal (Dingwall and Laskey, 1991; Yamasaki and Lanford, 1992). Inclusion of both region 40–57 (SKRLSSRARKRAAKRRLG) and 99–110 in the fusion protein resulted in nucleolar and nuclear localization while 40–57 alone was not sufficient for transport of β-galactosidase to the nucleolus (Valdez *et al.*, 1994). These data showed that the region comprising residues 40–57 represents a nucleolar localization signal that requires presence of the nuclear localization signal (99–110). The 40–57 domain is highly conserved between the human p120 and the mouse homolog; the 15 central residues (KRLSSRARKRAAKRR) are identical (Valdez *et al.*, 1994).

Domain(s) interacting with RNA. Protein p120 contains a potential arginine-rich RNA binding motif (ARM-motif, Burd and Dreyfuss, 1994). Interestingly, this motif is part of the nucleolar localization signal. To determine whether p120 binds to RNA, protein p120 was extracted from nucleoli of human cells (HeLa cells) with 10 mM Tris-HCl (pH 8.0)/10 mM KCl/0.5 mM $MgCl_2$/0.15% deoxycholate. This buffer has been used previously to extract pre-ribosomal RNP particles or monomer proteins from nucleoli (Freeman *et al.*, 1988) and, without deoxycholate, has been used as starting material for extraction of pre-ribosomal particles of snRNP particles (Prestayko *et al.*, 1974; Auger-Buendia and Longuet, 1978). Monoclonal antibodies (MoAbs) to p120 (Freeman *et al.*, 1988), B23 and C23 (Spector *et al.*, 1984) and polyclonal serum to fibrillarin (purchased from Sigma) were used to detect specific proteins. Immunodetection by ELISA assay showed that a major fraction of p120 sedimented with 60–80 S particles when nucleolar extracts were subjected to sucrose gradient centrifugation (Figures 3, 4). In addition, a minor, more slowly sedimenting p120 fraction was observed at 20–40 S.

For comparison, fibrillarin, a nucleolar protein associated with U3, U8 and U13 snRNP's sedimented at 20–30 S in monomer particles and a minor fraction sedimented at 60–80 S with preribosomal particles (Figure 3) in agreement with previous observations (Tyc and Steitz, 1989). Two nucleolar proteins present in relatively high abundance, C23 (nucleolin) and B23 (nucleophosmin), also differed from p120 in their sedimentation patterns on sucrose gradients: most of C23 sedimented near the top of the gradient (5–10 S); B23 sedimented slightly faster possibly as a result of its proposed native hexamer structure (Yung and Chan, 1987) at 10–15 S (Figure 3, 4). The relative high abundance nucleolar proteins C23 and B23 represent major Coomassie blue bands near the top of the gradient (Figure 4a). Protein p120 was not detectable in the Coomassie blue stained pattern in the 60–80 S region (fractions 2–4; Figure 4a) but was found in a peak in lane 2 on Western blots (Figure 4b) (corresponding to fractions 3 and 4, Figure 4a).

Treatment of rapidly sedimenting fractions containing p120 with buffers of increasing ionic strength shifted the p120 sedimentation pattern towards the top of the gradient,

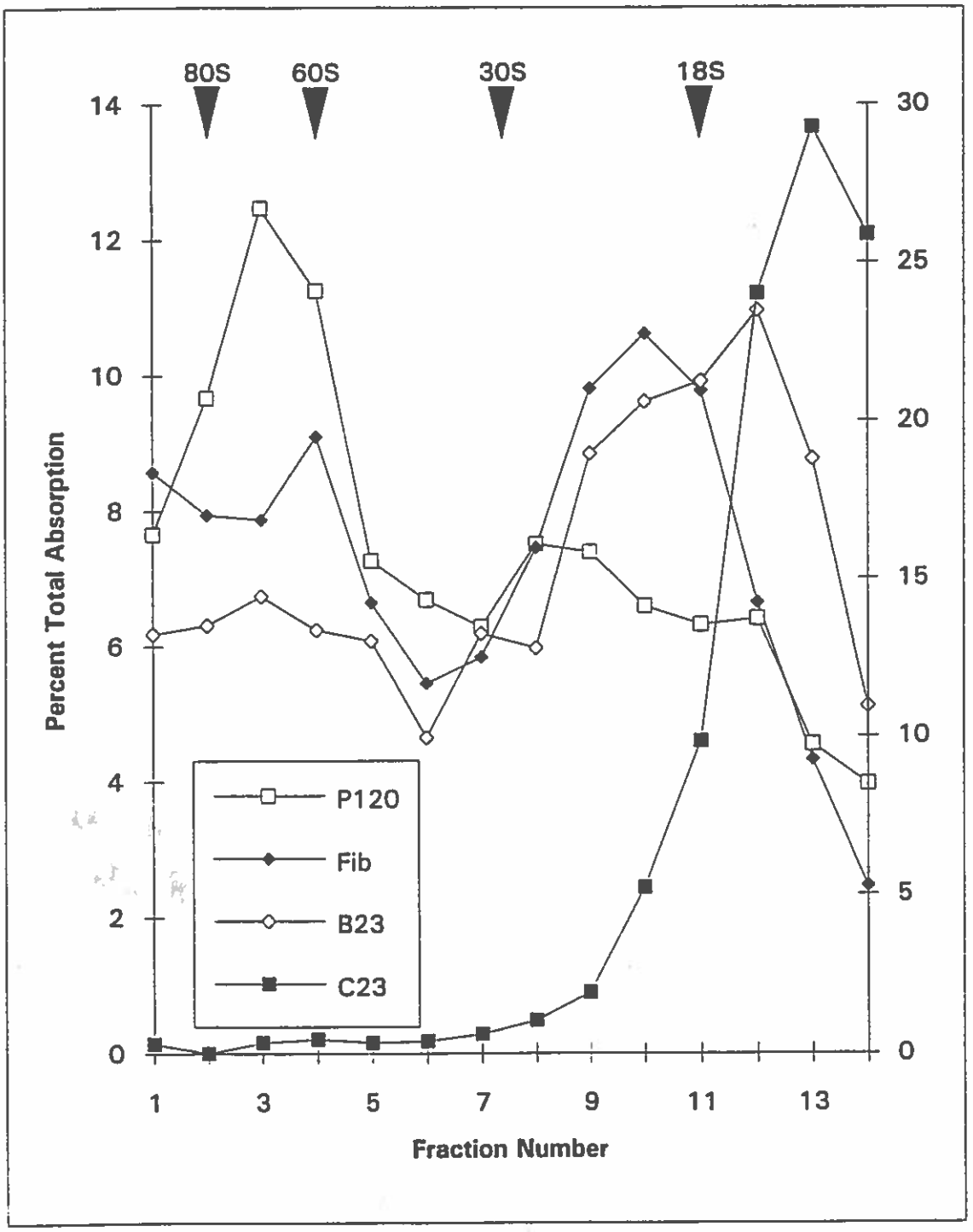


FIGURE 3. Fractionation of nucleolar extract by sucrose density gradient ultracentrifugation. The nucleolar extract was centrifuged for 17 hr in a 5-45% sucrose gradient at 25,000 rpm in a SW41 rotor. Fraction numbers are from bottom to top of the gradient. 50 ul aliquots of each fraction were analyzed for immunoreactivity by ELISA assays using MoAbs to p120, C23 and B23 and human autoimmune patient sera to fibrillarin.

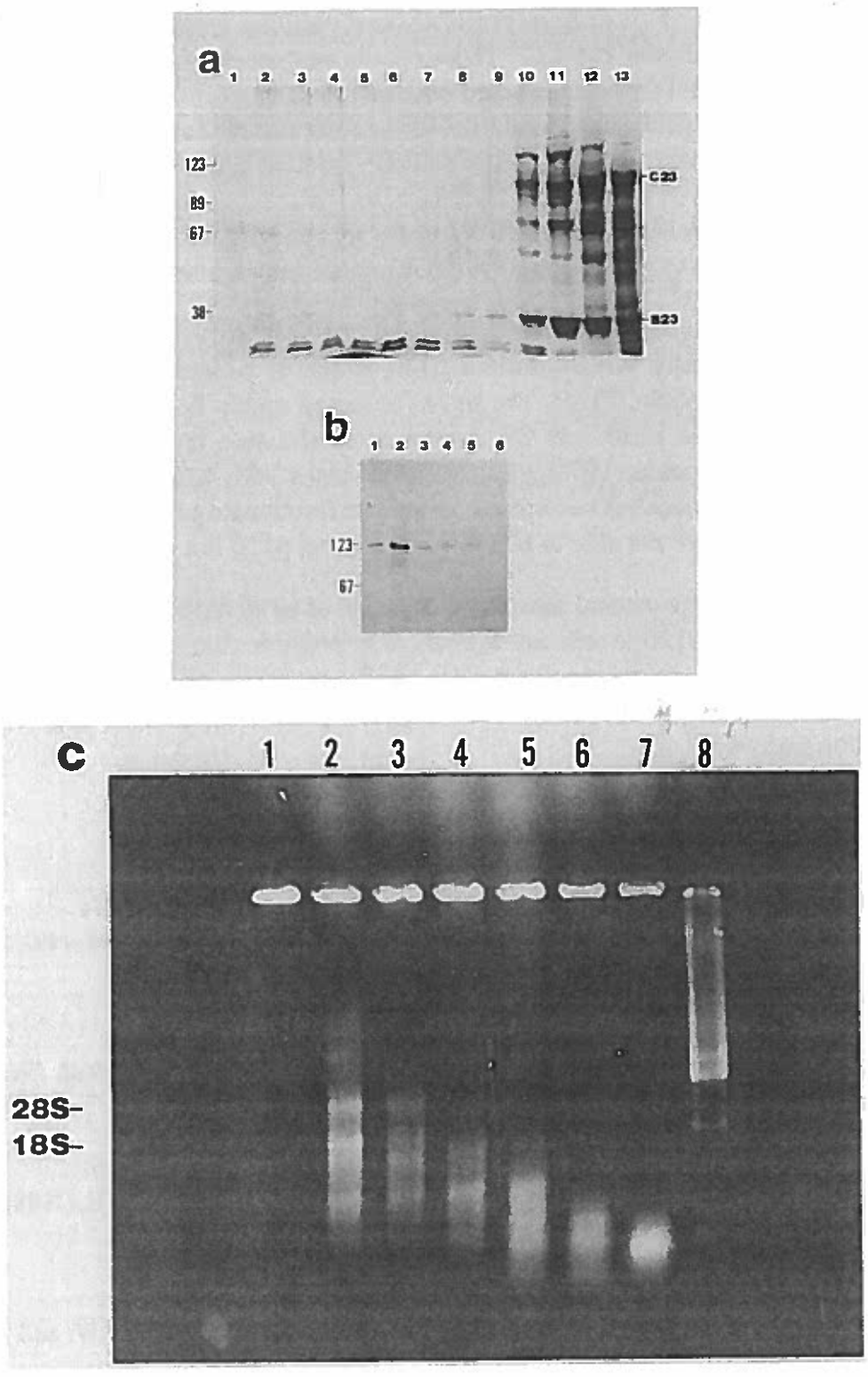


FIGURE 4. Electrophoretic separation of nucleolar extract fractions from sucrose density gradients. a) fractions were run on 7.5% acrylamnide gel and stained with Coomassie blue; lane 1, bottom of the gradient; lane 13, top of the gradient, b) Western immunoblot of fractions run on 7.5% acrylamide gel: each fraction represents the pool of 2 neighboring sucrose gradient fractions, lane 1 containing the bottom 2 fractions of the gradient, lane 2 fractions 3 and 4, etc. c) RNA was isolated from two-fraction pools by phenol/chloroform extraction, electrophoresed on 1% agarose gels and stained with ethidium bromide. Lanes are as in b), lane 8, total cellular RNA as source of 28 S and 18 S markers.

reflecting dissociation of p120 from pre-ribosomal particles (data not shown). Complete dissociation was found with 1M KCl. These results suggest that in part the association of p120 with pre-ribosomal particles is based on ionic bonds.

A major fraction of p120 also was shifted towards the top of the gradient when nucleolar extracts were treated with RNAse before sucrose gradient ultracentrifugation suggesting that p120 was bound to RNA in the 60-80 S particles (data not shown). DNAse had no effect on p120 sedimentation behavior which indicates that p120 does not interact with DNA. Studies are in progress to characterize the RNA regions which interact with p120.

RNA methylase domain. A recent computer-based search revealed a potential rRNA methyltransferase domain within protein p120 which is conserved in bacterial rRNA methyltransferases (Koonin, 1994). The most conserved region between bacterial proteins and human and mouse p120 was the S-adenosylmethionine binding motif typical for methyltransferases (Koonin, 1994) localized at residues 388-402 of p120. Utilizing hydroxylapatite or phosphocellulose chromatography to fractionate p120-containing nucleolar extracts, to date we were not able to obtain evidence that p120 is a methylase.

Conclusions. Although several functional domains of p120 have been characterized, the physiological role of p120 is still not known. It is possible that p120's association with pre-rRNP particles reflects its involvement in rRNP processing or maturation. Phosphorylation by specific kinases at specific sites suggests p120 may contain targets of signal transduction pathways.

References

1. Auger-Buendia, M.-A. and Longuet, M. (1978) *Eur. J. Biochem. 85:*105-114.
2. Boyle, W.J., Geer, P.V.D. and Hunter, T. (1991) *Methods Enzymol. 201:*110-149.
3. Burd, C.G. and Dreyfuss, G. (1994) *Science 265:*615-621.
4. Cardellini, E. and Durban, E. (1993) *Biochem. J. 291:*303-307.
5. Dingwall, C. and Laskey, R.A. (1991) *Trends Biochem Sci. 16:*478-481.
6. Duplay, P., Szmelcman, S., Bedouelle, H. and Hofnung, M. (1987) *J. Mol. Biol. 194:*663-673.
7. Fonagy, A., Henning, D., Jhiang, S., Haidar, M.A., Busch, R.K., Larson, R.G., Valdez, B. and Busch, H. (1989) *Cancer Commun. 1:*243-251.
8. Freeman, J.W. and Bondada, V. (1990) *Proc. Am. Assoc. Cancer Res. 31:*261.
9. Freeman, J.W., Busch, R.K., Gyorkey, F., Gyorkey, P., Ross, B.E. and Busch, H. (1988) *Cancer Res. 48:*1244-1251.
10. Koonin, E.V. (1994) *Nucleic Acids Res. 22:*2476-2478.
11. Laemmli, U. (1970) *Nature (London) 227:*680-685.
12. Larson, R.G., Henning, D., Haidar, M.A., Jhiang, S., Lin, W.L., Zhang, W.W. and Busch, H. (1990) *Cancer Commun. 2:*63-67.
13. Lawrence, C.B., Shalom, T.Y. and Honda, S. (1989) *Eugene: A Software Package for Nucleotide and Protein Sequence Analysis for UNIX Systems.* Molecular Biology Information Resource, Department of Cell Biology, Baylor College of Medicine, Houston, TX.
14. Muramatsu, M., Hayashi, Y. and Onishi, T. (1974) *Exp. Cell. Res. 88:*345-351.
15. Perlaky, L., Valdez, B.C., Busch, R.K., Larson, R.G., Jhiang, S., Zhang, W.W., Brattain, M. and Busch, H. (1992) *Cancer Res. 52:*428-436.
16. Prestayko, A.W., Klomp, G.R., Schmoll, D.R. and Busch, H. (1974) *Biochemistry 13:*1945-1951.

17. Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) *Molecular Cloning. A Laboratory Manual 2nd ed.,* Cold Spring Harbor Press.
18. Spector, D.L., Ochs, R.L. and Busch, H. (1984) *Chromosoma 90:*139-148.
19. Towbin, H., Staehelin, T. and Gordan, J. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76:*4350-4354.
20. Tyc, K. and Steitz, J.A. (1989) *EMBO J. 8:*3113-3119.
21. Valdez, B.C., Busch, R.K., Larson, R.G. and Busch, H. (1990a) *Cancer Res. 50:*2704-2707.
22. Valdez, B.C., Busch, R.K. and Busch, H. (1990b) *Biochem. Biophys. Res. Commun. 173:*423-430.
23. Valdez, B.C., Perlaky, L., Saijo, Y., Henning, D., Zhu, C., Busch, R.K., Zhang, W.W. and Busch, H. (1992) *Cancer Res. 52:*5681-5687.
24. Valdez, B.C., Perlaky, L., Henning, D., Saijo, Y., Chan, P.K. and Busch, H. (1994) *J. Biol. Chem. 269:*23776-23783.
25. Yamasaki, L. and Lanford, R.E. (1992) In: *Nuclear Trafficking* (C.M. Feldherr, ed), Academic Press, N.Y., pp. 121-174.
26. Yung, B.Y.M. and Chan, P.K. (1987) *Biochim. Biophys. Acta 925:*74-82.

Physiol. Chem. Phys. & Med. NMR (1995) 27:313-320

Protein-DNA Interactions at the Nuclear Scaffold Attachment Regions of DNA Loops

Anna Ferraro, Margherita Eufemi, Laura Cervoni, Fabio Altieri and Carlo Turano

Dipartimento di Scienze Biochimiche 'Alessandro Rossi Fanelli' e Centro di Biologia Molecolare del CNR, Università la Sapienza, 00185 Roma, Italy

Abstract: DNA in cell nuclei is organized in large loops, which are formed by the binding of DNA to proteins present in a nuclear structure called matrix or scaffold. There is ample evidence that these interactions have not only a structural role, but also a functional one. Studies of these interactions have so far been carried out mainly *in vitro*. Their relevance, therefore, for the *in vivo* situation is not proven. We have analyzed the DNA-protein interactions directly in the intact nucleus by means of cross-linking reactions. Cross-linking by UV irradiation and by cis-diamine dichloro platinum (II) have been performed on nuclei from chicken liver and compared. The platinum complex has been found to be more efficient. The proteins complexed to DNA have been isolated and analyzed, and have been found to be enriched in species present in the nuclear scaffold, independently from its method of preparation.

THE NUCLEAR MATRIX or scaffold is a structural protein meshwork of the cell nucleus which is thought to be the site of many important processes, as for example replication and transcription of DNA, and splicing and transport of RNA (1, 2). The nuclear scaffold also constitutes the point of attachment of large loops of chromatin, which seem to be independent transcription domains, regulated by enhancer elements located in proximity of, or coinciding with, the attachment regions of DNA (for a recent review, see reference 3). These nuclear matrix- (MAR) or nuclear scaffold- (SAR) associated regions of DNA, and the proteins interacting with them, have been the subject of intensive investigations in the last decade, in an attempt to understand these important structural and functional features of the nucleus. Many nuclear scaffold proteins have been identified as capable of specific binding to SAR fragments and also a number of SARs have been cloned and sequenced, so that some of their more common sequence features are well known (reviewed in reference 4).

However, some caution has been expressed on interpretation of the data obtained so far on the SAR-nuclear matrix interactions (1, 5). In fact, SARs are identified either by preparing the nuclear scaffold and isolating the DNA fragments which remain bound to the

insoluble scaffold structure or by testing fragments of DNA, already characterized, for recognition and binding by a scaffold preparation. In either case the nucleus must be broken and a preparation of a nuclear scaffold must be carried out. But the scaffold is not a well defined biological structure, because its composition is strongly dependent on the method used for its preparation. In fact matrix and scaffold are used to indicate two different preparations of the nuclear skeleton (although only the term 'nuclear scaffold' will be used hereafter). Even its very existence has been put in doubt, and, apart from its peripheral moiety, *i.e.* the nuclear lamina, its preparation may require a poorly defined stabilization step (6). Once the significance of any preparation of nuclear scaffold is questioned, the identification of SARs and of SAR-binding proteins is also doubtful. The possibility of artifacts originates from the experimental conditions required to remove the salt-soluble nuclear proteins and most of the DNA. To obtain that, either saline solutions (usually at high concentration) or detergents are used (together with DNAases and RNAases) all of which are likely to disturb the interactions existing *in vivo*.

We considered that it could be profitable to tackle this problem with a technique by which the DNA-protein interactions were stabilized in the intact nucleus, i.e. before any artifactual interaction could take place and before any real interaction could be lost. Nucleic acid-protein cross-linking methods have been described and widely used, and have helped to clarify complex biological structures, such as ribosomes. The most commonly used cross-linking methods are UV irradiation, either by usual UV sources (7) or by laser (8), formaldehyde (9) and heavy metals (10). A very ingenious method proposed by Mirzabekov and coworkers (7) exploited the selective cutting of DNA in correspondence of methylated guanine residues, with a subsequent cross-linking of proteins placed nearby. Formaldehyde has been used particularly to study histone-DNA interactions, since it is a very efficient cross-linker for these basic nuclear proteins (9). Hnilica and coworkers (10) pioneered the study of the cross-linking action of heavy metals and metal complexes, and showed that in this way a variety of non-histone proteins could form stable complexes with DNA. They also showed that proteins from the nuclear scaffold could be found among the proteins cross-linked to DNA in this way (11, 12). Cross-linking of these proteins by UV irradiation has also been reported (13).

We report here a study of the interactions between DNA and proteins in intact nuclei by UV irradiation and by the use of cis-diamine dichloro platinum (cis-DDP) which has been shown (10, 14) to be a very efficient cross-linker of non-histone nuclear proteins, and has also been previously used by us to compare the DNA-protein interactions in the nuclei from the liver of different animal species (15, 16). By assuming that the most abundant non-histone proteins interacting with DNA should be those having the role of anchoring the DNA loops to any structure existing *in vivo* inside the nucleus, we were expecting to find proteins of the nuclear scaffold, prepared by conventional methods, to be the main components of the cross-linked species. This expectation was, indeed, met by the results of the experiments.

Materials and Methods

Nuclei were prepared from chicken liver according to Blobel and Potter (17), and nuclear scaffolds were prepared either according to Berezney and Coffey (18) or according to Izaurralde *et al.* (19).

The cross-linking reaction by UV irradiation was carried out on intact nuclei according

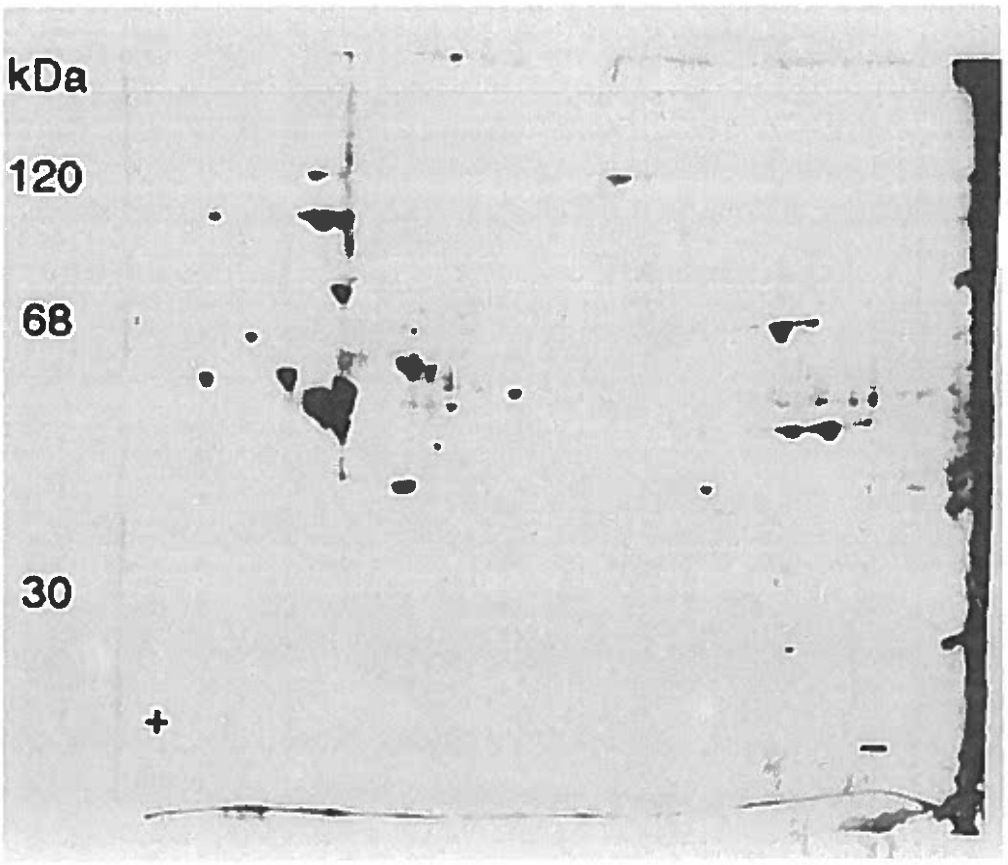


FIGURE 1. Two dimensional gel electrophoresis of the proteins isolated from the DNA-proteins complexes formed by cis-DDP.

to Mirzabekov *et al.* (7). The cross-linked nuclei were lysed with 0.5% SDS, the DNA was sheared by sonication, and the clear solution was fractionated through a Sephacryl 400 (Pharmacia) column. The fractions containing DNA and DNA-protein complexes were collected and treated with two volumes of ethanol. The precipitate was exhaustively digested with benzonase (Merck), and the residual proteins were analyzed by two-dimensional gel electrophoresis according to O'Farrell (20).

The cross-linking reaction with cis-DDP (Sigma) was performed on the intact nuclei according to Ferraro *et al.* (15) and the proteins were isolated from the complexes with DNA by the hydroxyapatite procedure (15). Nuclei were reacted with iodoacetamide according to Kaufmann and Shaper (21). The gels were stained by Coomassie Blue, dried and analyzed by a Bio-Rad 620 video densitometer. Comparisons of gels were made by the use of the 2D Analyst II program (Bio-Rad).

Results

Nuclei purified from chicken liver were subjected to UV irradiation or to the action of cis-DDP, as described in the preceding section, and the proteins cross-linked to DNA were isolated, freed from nucleic acid and analyzed by SDS-gel electrophoresis. The results are shown in Figures 1 and 2. It can be easily seen that cis-DDP is a more efficient cross-linking

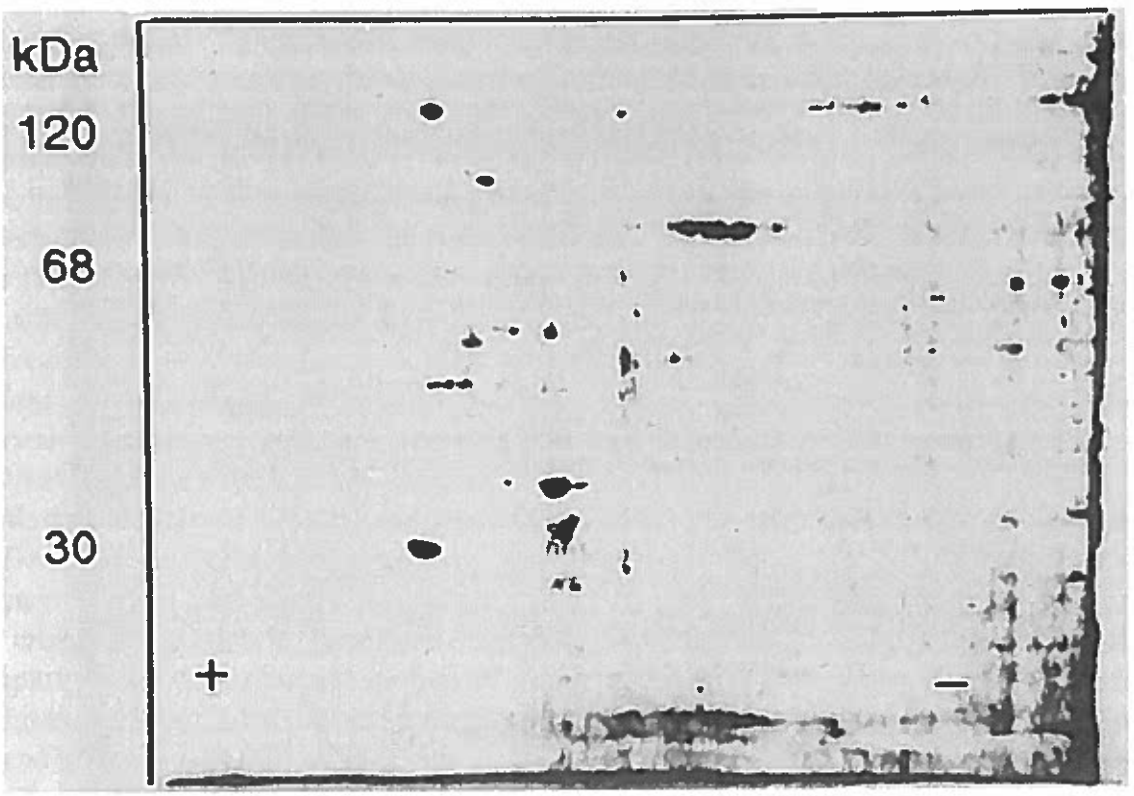


FIGURE 2.. Two dimensional gel electrophoresis of the proteins isolated from the DNA-proteins complexes formed by UV irradiation.

agent than UV. Furthermore, cis-DDP appears to be selective for non-histone proteins, as reported earlier by Filipski *et al.* (14), while histones are subjected to cross-linking by UV irradiation, as demonstrated by mono-dimensional SDS-gel electrophoresis (data not shown). The absence of the abundant histones among the cross-linked proteins is a clear advantage, when the aim is to isolate and analyze non-histone proteins. Cis-DDP was therefore chosen for a detailed analysis of the DNA-proteins interactions in chicken liver nuclei.

Before the cis-DDP-driven cross-linking reaction, nuclei were treated with iodoacetamide, in order to block all reactive sulfhydryl groups of the nuclear proteins, and, in particular, those of the nuclear scaffold. As Kaufmann and Shaper pointed out (21), free sulfhydryl groups of the scaffold easily undergo an oxidation during the scaffold preparation, and this could lead to the finding, among the cross-linked species, of proteins which are not interacting directly with DNA, but only indirectly through protein-protein associations mediated by disulfide bonds.

To detect among the proteins derived from the complexes those which could be found also in the nuclear scaffold, the latter were prepared according to Berezney and Coffey (18) by high salt extraction of nuclei and according to Laemmli (19) by detergent extraction, and they were also analyzed by two-dimensional electrophoresis (Figure 3 and 4). When a computer-aided comparison of the patterns was performed, it was found that most of the cross-linked proteins were indeed components of the nuclear scaffold (Figure 5). At this

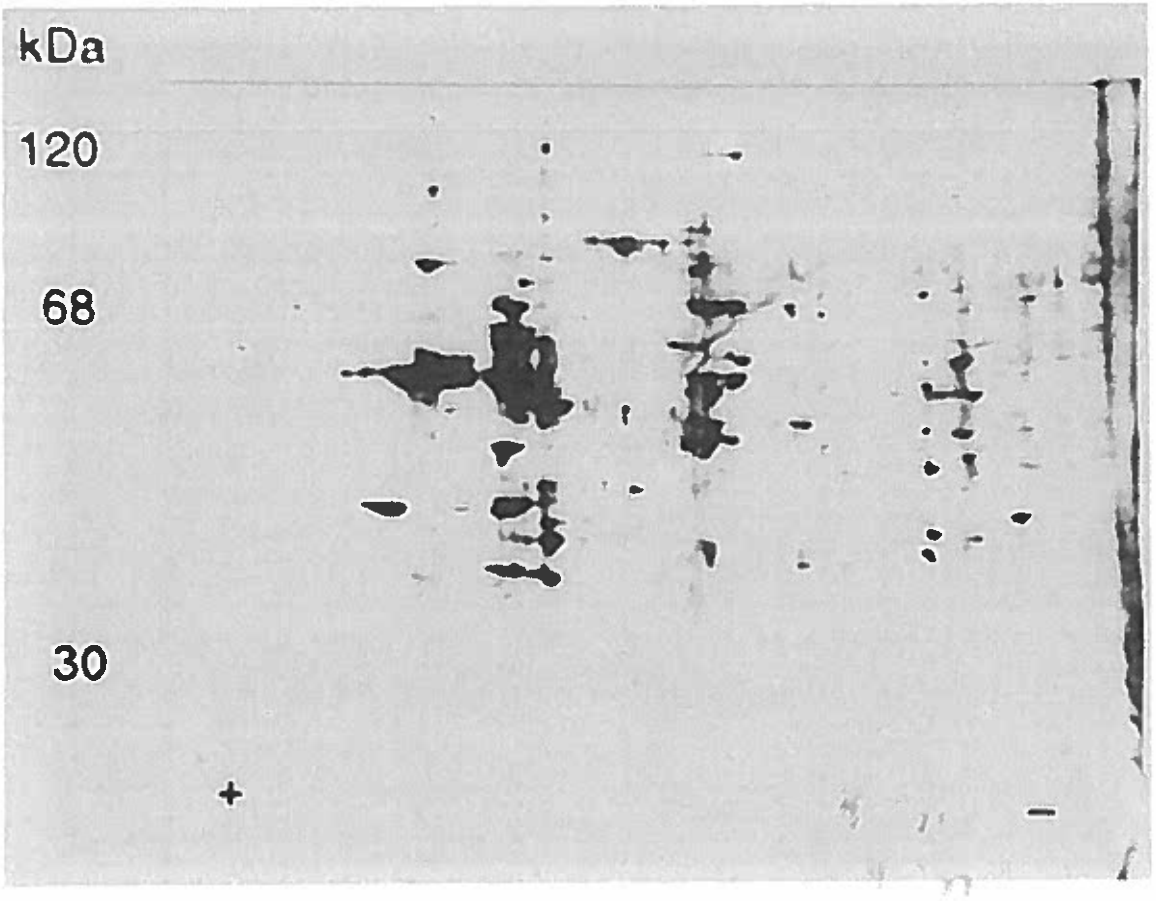


FIGURE 3. Two dimensional gel electrophoresis of the proteins of the nuclear scaffold prepared by high salt extraction (18).

stage no attempt was made to identify the scaffold proteins involved in interactions with DNA.

Discussion

The two cross-linking agents which we have used, namely UV irradiation and cis-DDP, when acted upon chicken liver nuclei were able to form a number of protein-DNA complexes, from which the protein components could be isolated and analyzed. However, at least half of the UV cross-linked protein species were different from those cross-linked by cis-DDP. This was not unexpected, because the amino acid side chains and the bases of the nucleic acid which are likely to participate in the cross-linking depend on the nature of the cross-linking agent used. They are probably represented by tyrosine and thymine when UV is employed, and by histidine or cysteine and guanine when cis-DDP is used. Therefore not all proteins located in proximity to DNA will react but only those in which a reactive nucleotide/amino acid pair is placed in a favorable position. Furthermore, in the case of cis-DDP the accessibility of the metal complex to the reactive site is also required. For these reasons the cross-linking experiments provide the evidence of the proximity of a certain protein species to DNA when the cross-linking takes place, but cannot rule out the proximity to DNA of a protein which does not become cross-linked.

As seen in Figures 1 and 2, cis-DDP seems more efficient than UV irradiation, at least on

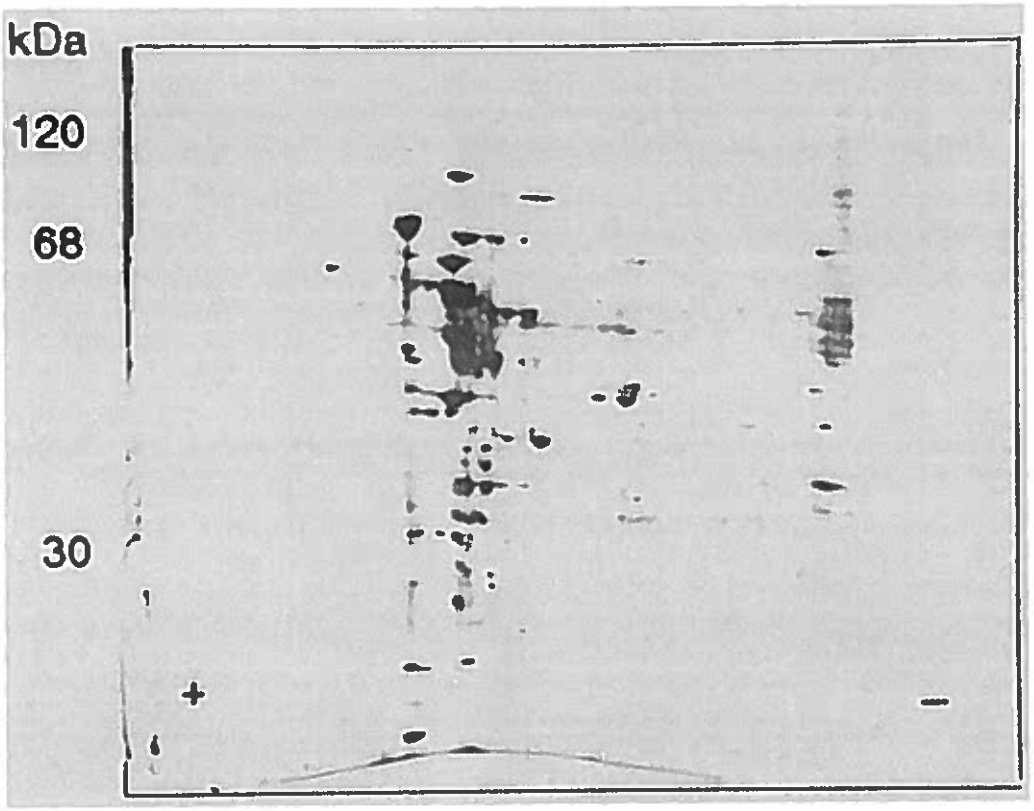


FIGURE 4. Two dimensional gel electrophoresis of the proteins of the nuclear scaffold prepared by detergent extraction (19).

chicken liver nuclei. Therefore, considering also the easiness of dissociation of the cis-DDP-induced complexes (14), we used this reagent for the investigation of the anchorage site of DNA to the nuclear scaffold.

The effect of blocking the free sulfhydryl groups of the nuclei with iodoacetamide before the reaction with cis-DDP can be evaluated by comparing the pattern of cross-linked proteins shown in Figure 1 with that obtained previously (16) without the use of the alkylating reagent. It can be seen that, although the two patterns are very similar, the use of the alkylating reagent leads to a decrease in the number of isolated proteins. This can be caused either by the blocking of some sulfhydryl groups required for the cross-linking reaction, or else by the lack of formation of some disulfide bridges between two nuclear proteins, one of which is the real DNA-binding protein, and the second which is located in the proximity of the first one. In either case, all the proteins shown in Figure 1 should represent species interacting directly with DNA.

The usefulness of this cross-linking technique for the detection of the anchorage points of DNA on the nuclear scaffold rests on the validity of the assumption that the major cross-linked protein species are represented by relatively abundant structural components of the nucleus, which should be found among the proteins of the nuclear scaffold. In fact Wedrychowski *et al.* (12) have described the presence of such proteins, identified by immunological criteria, in cross-linked complexes. In order to evaluate the relative abundance, and possibly the nature, of nuclear scaffold proteins among the cross-linked species,

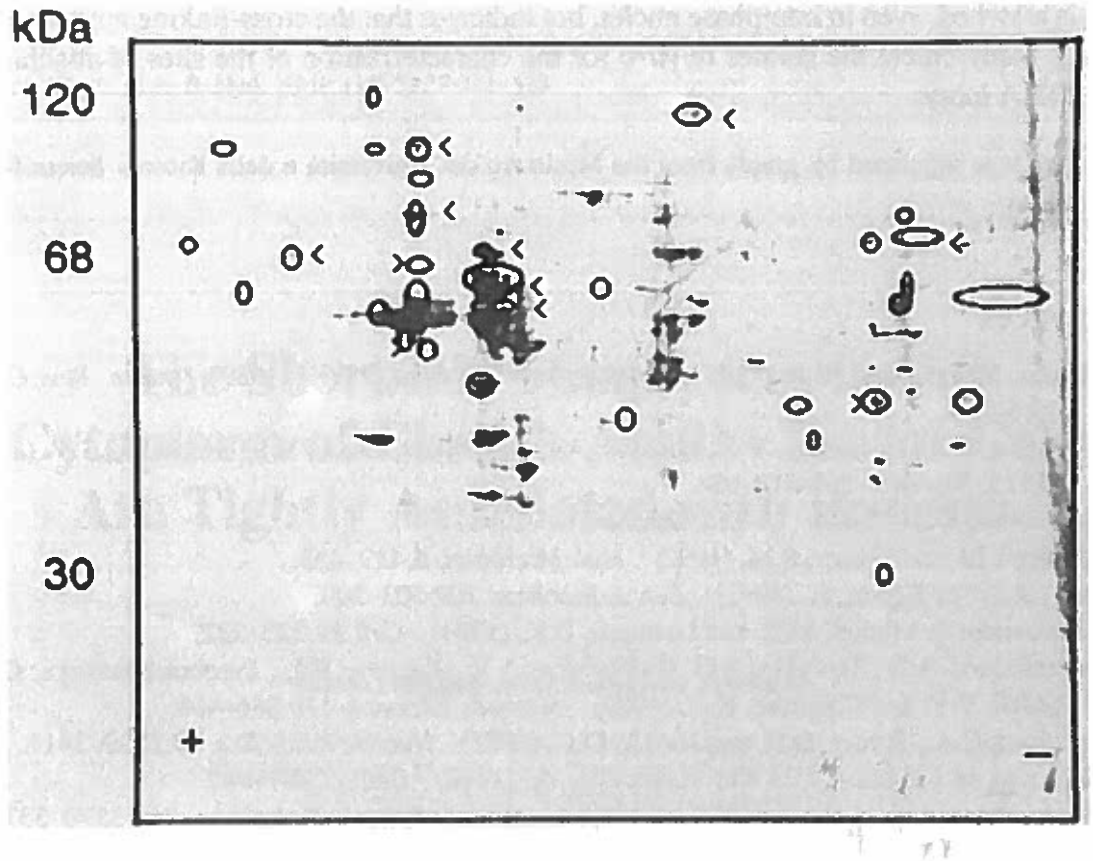


FIGURE 5. Two dimensional gel electrophoresis of the proteins of the nuclear scaffold prepared by high salt extraction (same as Figure 3), which shows superimposed the proteins cross-linked to DNA by cis-DDP (same as Figure 1, but with the protein spots shown as circles). The arrowheads indicate the cross-linked protein species present in both scaffold preparations (i.e. the common proteins in Figures 1, 3 and 4).

we have previously compared the electrophoretic patterns of cross-linked proteins to those of the nuclear scaffold prepared according to Kaufmann and Shaper (21). However, since the composition of the scaffold varies with the method employed for its preparation, it was essential to extend this comparison to other common types of preparation.

Shown in Figures 3 and 4 are the two-dimensional electrophoretic patterns of the proteins from the nuclear scaffold prepared according to Berezney and Coffey (18) by high salt extraction and from that prepared according to Izaurralde *et al.* (19) by lithium-diiodo salicylate extraction. The two patterns are by no means identical, although a careful analysis reveals that they share many common spots.

A comparison of these patterns with that exhibited by the cross-linked proteins revealed that most of these proteins could be found among the species present in nuclear scaffold preparations, and particularly among the species present both in high salt and in detergent preparations (Figure 5). It can be noticed that the nuclear scaffold proteins with a low isoelectric point and with molecular masses ranging from about 45 to 100 kDa appear to be enriched in components interacting with DNA. The acidic nature of these proteins suggests that a specific interaction is taking place, rather than a simple electrostatic binding of a positively charged protein to DNA.

These results not only support the existence *in vivo* of an intranuclear structure to which DNA is attached, even in interphase nuclei, but indicates that the cross-linking approach can usefully complement the studies *in vitro* for the characterization of the sites of attachment of the DNA loops.

This work was supported by grants from the Ministero del'Università e della Ricerca Scientifica e Tecnologica.

References

1. Gasser, S.M., Amati, B.B., Cardenas, M.E. and Hofmann, J.F.X. (1989) *Intern. Rev. Cytol. 19:*57-96.
2. Georgiev, G.P., Vassetzky, Y.S., Lochnik, A.N., Chernokhvostov, V.V. and Razin, S.V. (1991) *Eur. J. Biochem. 200:*613-624.
3. Getzenberg, R.H. (1994) *J. Cell. Biochem. 55:*22-31.
4. Roberge, M. and Gasser, S.M. (1992) *Mol. Microbiol. 6:*419-423.
5. Jack, R.S. and Eggert, H. (1992) *Eur. J. Biochem. 209:*503-509.
6. Mirkovitch, J., Mirault, M.E. and Laemmli, U.K. (1984) *Cell 39:*223-232.
7. Mirzabekov, A.D., Bavykin, S.G., Belyavsky, A.V., Karpov, V.L., Preobrazhenskaya, O.V., Shick, V.V. and Ebralidse, K.K. (1989) *Methods Enzymol. 170:*386-408.
8. Harrison, C.A., Turner, D.H. and Hinkle, D.C. (1982) *Nucleic Acids Res. 10:*2399-2414.
9. Solomon, M.J., Larsen, P.L. and Varshavsky, A. (1988) *Cell 53:*937-947.
10. Wedrychowski, A., Schmidt, W.N. and Hnilica, N.S. (1986) *J. Biol. Chem. 261:*3370-3376.
11. Olinski, R., Wedrychowski, A., Schmidt, W.N., Briggs, R.C. and Hnilica, L.S. (1987) *Cancer Res. 47:*201-205.
12. Wedrychowski, A., Bhorjee, J.S. and Briggs, R.C. (1989) *Exp. Cell Res. 183:*376-387.
13. Boulikas, T. (1986) *Biochem. Cell Biol. 64:*474-484.
14. Filipski, J., Kohn, K.W. and Bonner, W.M. (1983) *FEBS Lett. 152:*105-108.
15. Ferraro, A., Grandi, P., Eufemi, M., Altieri, F., Cervoni, L. and Turano, C. (1991) *Biochem. Biophys. Res. Comm. 178:*1365-1370.
16. Ferraro, A., Grandi, P., Eufemi, M., Altieri, F. and Turano, C. (199_) *FEBS Letters 307:*383-385.
17. Blobel, G. and Potter, V.R. (1966) *Science 154:*1162-1165.
18. Berezney, R. and Coffey, D.S. (1974) *Biochem. Biophys. Res. Commun. 178:*1365-1370.
19. Izaurralde, E., Mirkovitch, J. and Laemmli, U.K. (1988) *J. Mol. Biol. 200:*11-125.
20. O'Farrell, P.H. (1975) *J. Biol. Chem. 250:*4007-4021.
21. Kaufmann, S.H. and Shaper, J.H. (1984) *Exp. Cell Res. 155:*477-495.

Physiol. Chem. Phys & Med. NMR (1995) 27:321-328

The Short DNA Sequences in the Cytoplasm of Ehrlich Ascites Tumor Cells Are Tightly Associated with Proteins

Ralf Hegger and Hinrich Abken

Klinik I für Innere Medizin, Lab. Tumorgenetik und Zellbiologie, Universität zu Köln, D-50924 Köln, Germany

Abstract: Mouse Ehrlich tumor cells harbor extrachromosomal DNA elements in a non-mitochondrial fraction of the cytoplasm (Abken *et al., Proc. Natl. Acad. Sci USA 90:* 6518-6522, 1993). The cytoplasmic DNA sequences constitute a distinct group of extrachromosomal genetic elements with common properties: (i) the DNA molecules are 50-500 bp in length and of linear configuration, (ii) most of the DNA sequences exhibit the potential to modulate the activity of a transcriptional promoter, and (iii) the DNA elements are preferentially found in tumor cells, not in cells with normal phenotype. Unexpectedly, the extrachromosomal DNA sequences are found to be tightly associated with at least three proteins (52 kD, 62 kD, 64 kD) forming DNA-protein (DNP) complexes. The DNA-protein interaction is stable during extraction with phenol and chloroform and during incubation with proteinase K and pronase P, in the presence of detergents (SDS, NP40), guanidine-HCl, high salt concentrations, and alkali. Hydrolysis of the DNA by DNAse I makes the proteins of the DNP complex accessible to proteolytic degradation. Western blot analyses imply that the proteins associated with the cytoplasmic DNA sequences are antigenically related to nucleomatrix proteins that are covalently bound to certain regions of chromosomal DNA. Covalent bonds between the cytoplasmic DNA and the polypeptides would explain the unexpected high stability of the cytoplasmic DNA-protein complexes in tumor cells.

A VARIETY OF GENETIC ALTERATIONS of chromosomal DNA are found in tumor cells, some of them are causally linked with the initiation of neoplastic transformation of the cell. Most of the genetic alterations, however, are considered to be a consequence of the genetic instability of the tumor cell genome. In addition to structural alterations of the chromosomal DNA, extrachromosomal DNA elements are monitored preferentially in tumor cells, e.g., small polydispersed circles and double minute chromosomes. Previously, we reported that the cytoplasm of established tumor cell lines harbors short DNA sequences that induce human lymphocytes to escape senescence and to proliferate unlimited after transfection *in vitro* ("immortalization") (Abken *et al.*, 1988). Approaches to isolate the

DNA with immortalizing activities resulted in the identification of a distinct population of extrachromosomal DNA molecules that can be isolated from a cytoplasmic lysate by equilibrium centrifugation in a neutral CsCl density gradient (Abken *et al.*, 1993). The immortalizing DNA copurified together with this type of extrachromosomal DNA sequences in fractions at high salt densities (1.85–1.88 g/cm^3). In contrast, no DNA could be monitored in the corresponding fractions of normal, pre-senescent fibroblasts. The cytoplasmic DNA of tumor cells was found to be 50–500 bp in length and of linear configuration. From Ehrlich ascites cells we cloned 25 different DNA sequences. One of them, EFC38 (372 bp), induces unlimited proliferation of human lymphocytes after transfection. None of the DNA sequences cloned harbor an open reading frame for protein translation. On the basis of their common properties these DNA sequences are proposed to represent a distinct group of extrachromosomal DNA elements with so far unknown function(s) in tumor cells (for review: Abken, 1995). One of the most intriguing properties of the cytoplasmic DNA elements is their sedimentation in fractions of about 1.86 g/cm^3 during equilibrium centrifugation in a CsCl gradient (Abken *et al.*, 1993). Banding of DNA in fractions of these high salt concentrations is unusual, since mammalian DNA purifies at 1.699–1.700 g/cm^3 in the case of nuclear DNA (Corneo *et al.*, 1968), or at 1.686–1.712 g/cm^3 in the case of satellite DNA (Jones, 1973). The cloned DNA sequences from fractions of high salt densities sediment in factions at 1.70 g/cm^3 as expected for naked, double-stranded DNA. Thus, it is unlikely that these DNA sequences persist as naked double-stranded DNA molecules in the cytoplasm of the tumor cells. The physical parameters suggest that the DNA may be partially single-stranded and/or be associated with RNA and/or metallo-proteins. Here we provide evidences that the cytoplasmic DNA is tightly associated with proteins that seem to be antigenically related to proteins of the nuclear matrix covalently bound to certain regions of the chromosomal genome.

Materials and Methods

Mouse Ehrlich ascites cells were cultured in Dulbecco's modified Eagle's medium (DME medium). Cytoplasmic DNA was isolated from cytoplasts as described (Abken *et al.*, 1988, 1993). Briefly, cytoplasts (10^{10}) were induced by incubation with cytochalasin B, separated from cells, resuspended in 500 µl of 50 mM Tris-HCl, pH 7.5, 10 mM EDTA, 1.5 mM $MgCl_2$, and lysed by repeated freezing and thawing. Cellular debris was sedimented at 1,000 × g, and the supernatant was centrifuged to equilibrium in a neutral CsCl gradient (1.70 g/cm^3) at 180,000 × g and 10ºC. Fractions at 1.85–1.89 g/cm^3 were dialyzed into 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, and subsequently incubated with 100 µg/ml of RNaseA for 1 h at 37ºC and with 0.1% SDS, 100 µg/ml proteinase K for 12 h at 60ºC. Finally, DNA was extracted with phenol and chloroform/isoamyl alcohol.

Chromosomal DNA associated with the nuclear matrix was separated from protein-free DNA by filtration through nitrocellulose (BA85, Schleicher and Schuell) as described (Juodka *et al.*, 1991).

Recombinant DNA techniques, hybridizations and Western blot analyses were performed according to standard methods (Sambrook *et al.*, 1989, Harlow and Lane, 1988).

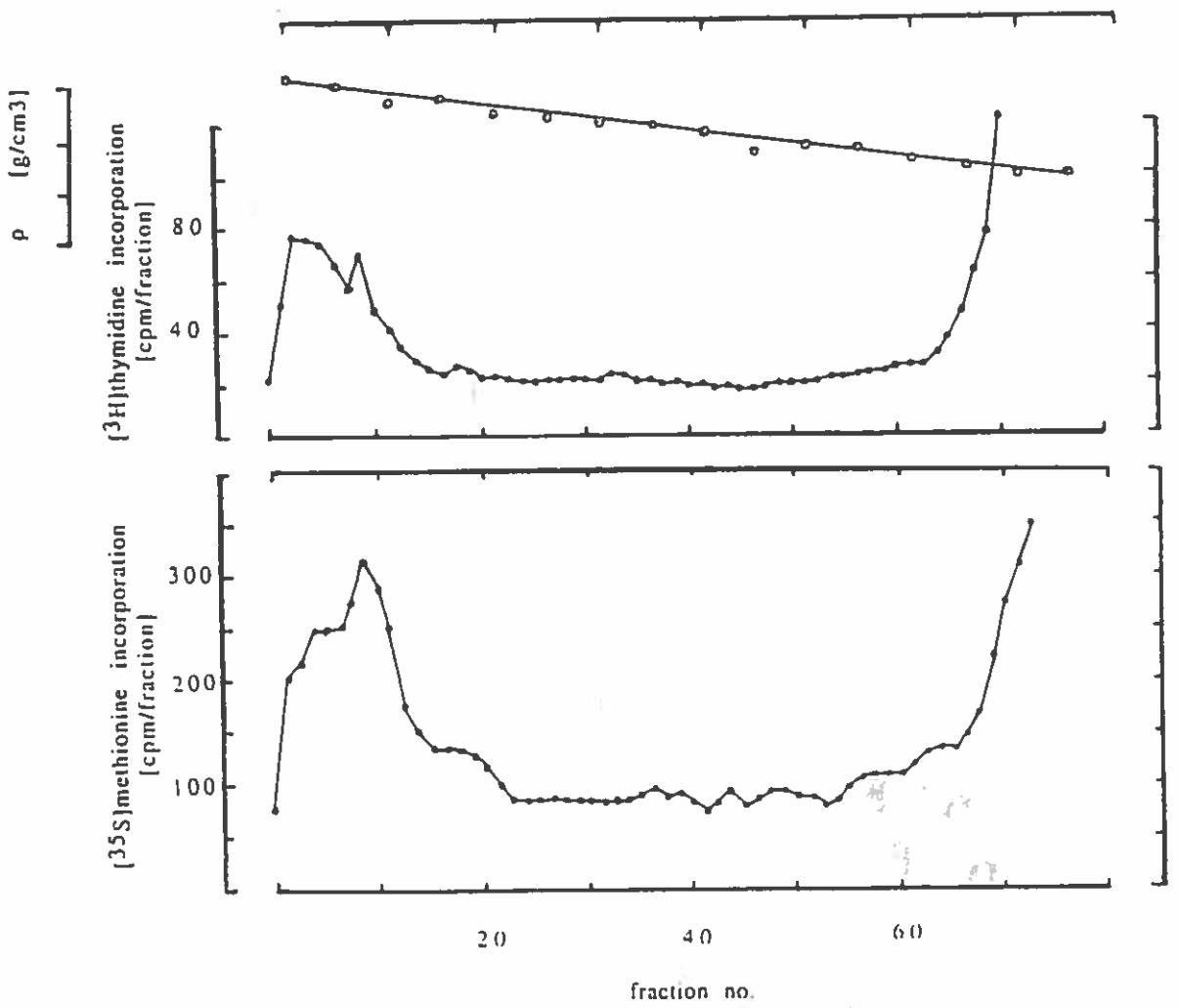


FIGURE 1. Equilibrium centrifugation of a cytoplasmic lysate from Ehrlich ascites cells in a CsCl density gradient.

Ehrlich ascites cells were cultured in the presence of [^{3}H]thymidine and [^{35}S]methionine, respectively, for 50 hr. The cells were lysed and the cytoplasmic lysate was centrifuged to equilibrium in a neutral CsCl density gradient as described (Abken *et al.*, 1993). DNA and proteins of each fraction were precipitated with trichloroacetic acid onto glass fiber filters and the incorporated radioactivity was determined by liquid scintillation spectrometry. The CsCl density was recorded by refractometry.

Results.

1. The cytoplasmic DNA in fractions of high salt density is associated with proteins.

Recently, we identified cytoplasmic DNA from Ehrlich ascites cells that sediments in fractions of high salt densities, i.e. 1.85–1.88 g/cm^3 (Abken *et al.*, 1993). To monitor whether proteins are associated with the extrachromosomal DNA, Ehrlich ascites cells were grown in the presence of [^{3}H]thymidine and [^{35}S]methionine, respectively. The cytoplasmic lysates were centrifuged to equilibrium in a neutral CsCl density gradient. As shown in Figure 1, [^{35}S]methionine labeled material co-sediments with the [^{3}H]thymidine labeled DNA in fractions of 1.85–1.88 g/cm^3. The DNA was recovered from fractions of high salt densities, dialyzed, extracted in phenol, and concentrated by precipitation with ethanol.

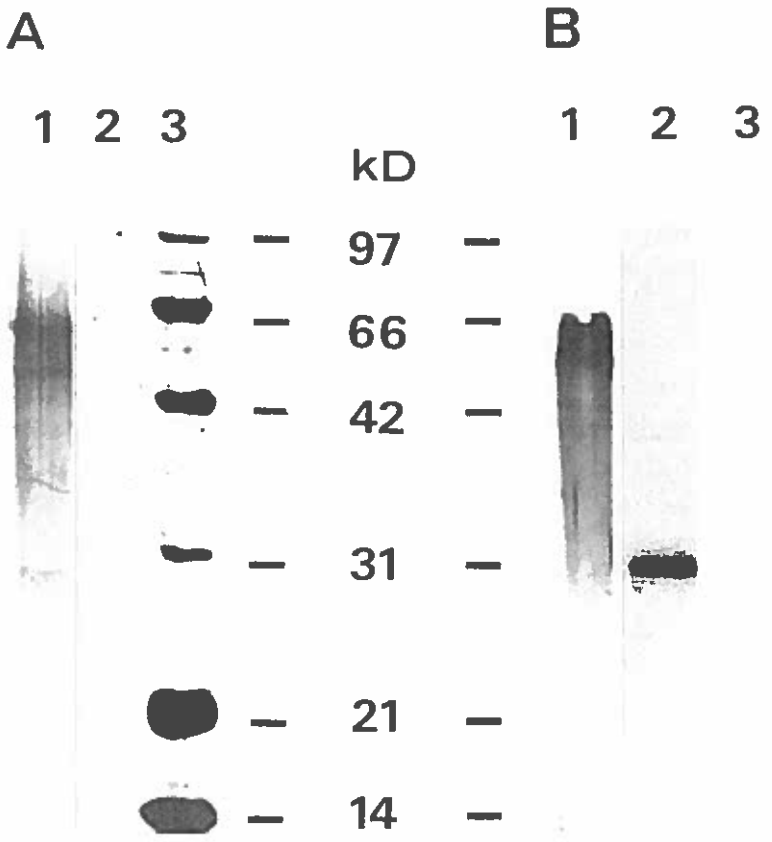


FIGURE 2. DNA-protein complexes isolated from the cytoplasm of Ehrlich ascites cells.

DNA-protein complexes were recovered from high density fractions of the cytoplasm of Ehrlich ascites cells, dialyzed, and subsequently treated with RNase A (100 mg/ml) for 1 h at 37°C and with proteinase K (100 mg/ml) for 12 h at 60°C as described.

(A) The DNA-protein complexes (2 µg DNA) (lane 1) and the cloned cytoplasmic DNA sequence EFC38 (372 bp) (2 µg) (lane 2) were separated electrophoretically in a SDS polyacrylamide gel. The proteins were visualized by silver staining. Lane 3 represents a protein marker as standard.

(B) The DNA of the DNA-protein complexes and of the EFC38 DNA was labeled with ^{32}P by polynucleotide kinase reaction and subsequently separated in a SDS polyacrylamide gel. The DNA was monitored by autoradiography of the gel.

Electrophoresis through an SDS polyacrylamide gel revealed at least three proteins (54 kD, 62 kD, 64 kD) that copurify with the cytoplasmic DNA of Ehrlich ascites cells (Figure 2A). This observation is unexpected since during isolation the DNA was extensively treated with proteases and repeatedly extracted in phenol.

To prove whether the DNA comigrates with the proteins in a denaturating gel, cytoplasmic DNA from fractions of high salt density was labeled with ^{32}P by polynucleotide kinase reaction and subjected to SDS polyacrylamide gel electrophoresis. The autoradiography revealed the labeled DNA comigrating with the same protein bands detected by silver staining (Figure 2B). We conclude that the extrachromosomal DNA in the cytoplasm of Ehrlich ascites cells is associated with proteins of an apparent molecular weight of 52 kD, 62 kD, and 64 kD forming DNA-protein (DNP) complexes. In contrast, the cloned cytoplasmic DNA sequences, e.g., 372 bp EFC38 DNA, are free of these proteins.

This type of DNA-protein complexes assumes very tight interactions between DNA and

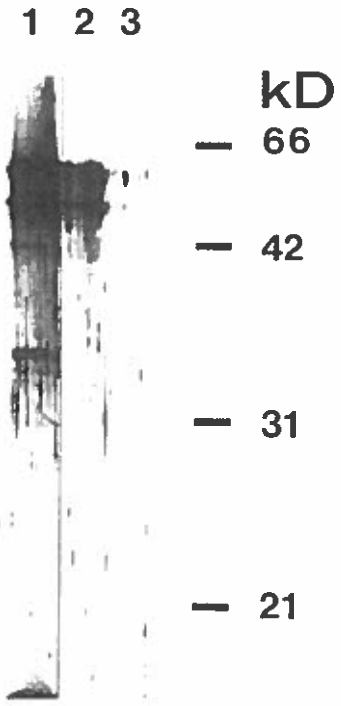


FIGURE 3. Western blot analysis of cytoplasmic and nuclear DNA-protein complexes.

Extrachromosomal DNA-protein complexes (2 µg DNA) from the cytoplasm of Ehrlich ascites cells (lane 1) and chromosomal DNA-protein complexes (2 µg DNA) (lane 2), that bind to nitrocellulose filters, and chromosomal protein-free DNA (2 µg) (lane 3), that passes the filter under these conditions, were separated electrophoretically in a denaturating SDS polyacrylamide gel and screened by Western blot techniques with the anti-DNP serum obtained after immunization with extrachromosomal cytoplasmic DNP complexes from Ehrlich cells.

polypeptides because the complex is obviously stable to high salt concentrations (i.e., 6.9 M CsCl in fractions at 1.86 g/cm^3) and to detergents (0.1% SDS). Noteworthy, the proteinaceous material associated with DNA in the DNA-protein complex is furthermore resistant to proteolytic degradation by incubation with proteinase K (100 µg/ml) and pronase P (100 µg/ml) for 1 h at 60ºC. After hydrolysis of the DNA by DNaseI (1 U/ml, 1 h, 37ºC) the proteins of the complex become susceptible to degradation by proteinase K. This suggests that the DNA may be arranged on the outside surface of the DNP complex protecting the proteins from proteolytic degradation.

In order to assay the stability of the DNA-polypeptide interaction, we incubated the cytoplasmic DNP complexes from Ehrlich ascites cells in the presence of 2 M NaCl, 6 M guanidine-HCl, and 0.1M NaOH, respectively. Equilibrium centrifugation in a CsCl gradient revealed that the DNA-protein complexes did not alter their sedimentation density by one of these treatments (not shown). This observation implies that the DNA-polypeptide association is even more stable than ionic or electrostatic interactions and may be due to covalent bonds between the DNA and polypeptides of the DNP complex.

2. The proteins of the cytoplasmic DNA-protein complexes are antigenically related to proteins of the nuclear matrix.

The high stability of the DNA-polypeptide interaction prompted us to compare the cytoplasmic DNA-protein complexes with complexes of certain chromosomal DNA regions tightly bound to non-histone proteins. The DNA-polypeptide interaction of the chromoso-

mal DNA-protein complexes are known to be salt-, detergent-, and alkali-stable due to phosphotriester bonds between hydroxyamino acid residues in peptides and the internucleotide phospates of the DNA (Juodka *et al.*, 1991). These chromosomal DNP complexes of high stability are considered to be part of the nuclear matrix. Nuclear DNP complexes were isolated by treatment of high molecular weight DNA with RNase, subsequently with SDS/proteinase K, and finally by repeated phenol extractions. DNA sequences tightly bound to nuclear peptides are specifically retained on nitrocellulose filters whereas protein-free DNA passes the filter (Neuer and Werner, 1985). By means of these properties, chromosomal DNA fragments tightly associated with polypeptides were isolated from protein-free fragments of Ehrlich ascites cells (kindly provided by Dr. D. Werner, Heidelberg). Electrophoresis in an SDS polyacrylamide gel demonstrated that the nuclear DNP complexes exhibit a similar pattern of protein bands as the cytoplasmic DNA-protein complexes (not shown). Recently, we generated an antiserum to this type of DNP complexes by immunization of rabbits with DNP complexes from the cytoplasm of Ehrlich ascites cells. Western blot analyses revealed that the antiserum to cytoplasmic DNP complexes detects the nuclear DNP complexes as well, whereas the protein-free chromosomal DNA is not recorded by the antiserum (i.e., Figure 3). We conclude that the cytoplasmic extrachromosomal and the nuclear chromosomal DNP complexes share antigenic determinants that are recognized by the same antiserum.

Discussion

Extrachromosomal DNA elements were previously isolated from the cytoplasm of Ehrlich ascites cells to identify the DNA with "immortalizing activity" that induces human lymphocytes to proliferate without limit after transfection *in vitro* (Abken *et al.*, 1988, 1993). Fractionation of the cytoplasm by equilibrium centrifugation in a CsCl gradient revealed a distinct group of DNA elements that have the following properties in common: (i) linear DNA molecules of 50–500 bp in length, (ii) association with proteins of about 52 kD, 62 kD, and 64 kD, (iii) sedimentation of the DNA-protein complex in fractions at 1.85–1.88 g/cm^3 of a CsCl density gradient, and (iv) location predominantly in a non-mitochondrial cytoplasmic fraction of the tumor cells (reviewed by Abken, 1995). Molecular cloning of the cytoplasmic DNA from Ehrlich ascites cells revealed 25 different DNA sequences (Abken *et al.*, 1993). In independent experiments each of these sequences was repeatedly cloned and always in full length. Therefore, it is very unlikely that the short DNA sequences cloned represent random fragments of chromosomal DNA generated during isolation and cloning procedures.

In order to isolate the DNA elements from the cytoplasm of Ehrlich cells it was essential to concentrate the DNP complex by CsCl density centrifugation. Consequently, the results reported here give no information about the size of the proteinaceous material additionally and loosely incorporated within the native complex. However, it is likely that the cytoplasmic complexes characterized here reflect the entire DNA-protein unit because of the following observations: (i) radiolabeling of the cytoplasmic DNA in a crude lysate by terminal transferase reaction prior to DNA isolation reveals DNA associated protein complexes that comigrate with the isolated DNP complexes in an SDS polyacrylamide gel, and (ii) the anti-DNP serum detects the same protein bands in crude cytoplasmic lysates as of isolated DNA-protein complexes.

Recently, we reconstituted DNA-protein complexes *in vitro* by incubation of the cloned cytoplasmic EFC38 DNA (372 bp) from Ehrlich ascites cells with a cytoplasmic protein extract. The reconstituted DNA-protein complexes exhibit the same properties as the DNP complexes isolated from the cytoplasm of the cells, i.e., sedimentation in fractions of high salt densities, binding to nitrocellulose filters, salt- and detergent-stable interaction with complex forming proteins. *In vivo,* after transfection of the protein-free EFC38 DNA into human lymphocytes, this type of salt- and detergent-stable DNA-protein complex is generated as well (Abken *et al.*, 1993).

Whereas the extrachromosomal DNP complexes are preferentially found in the cytoplasm, nuclear chromosomal DNA of Ehrlich tumor cells was shown to harbor certain regions that are tightly associated with non-histone proteins as well (for review: Werner *et al.*, 1988). These DNA-protein complexes of chromosomal DNA are stable in the presence of detergents, of high salt concentrations, and of alkali (Neuer and Werner, 1985). Proteins of the nuclear DNP complexes co-migrate with the proteins of the cytoplasmic DNP complex under denaturating conditions (cf. Figure 3). Western blot analyses now revealed that the nuclear and cytoplasmic DNA-protein complexes from Ehrlich ascites cells share antigenic determinants, that are recognized by the anti-cytoplasmic DNP serum. This observation suggests that the proteins of the cytoplasmic and nuclear DNP complexes may be identical or belong to a highly conserved family of non-histone proteins tightly associated with DNA. This assumption is supported by the observation that cytoplasmic DNP complexes with the same properties are found in mouse (L929, Ag8.653) and human (MCF-7, HT-29, L428) tumor lines and the nuclear DNP complexes in mammalia (Krauth and Werner, 1979; Dodnar *et al.*, 1983; Avramova and Tsanev, 1987; Razin *et al.*, 1988), in insect cells (Plagens, 1978), and in plant cells as well (Capesius *et al.*, 1980; Avramova *et al.*, 1988). Chromosomal DNA isolated from Ehrlich ascites cells by prolonged alkaline cell lysis and phenol extraction was analyzed with respect of the linking groups between peptides and polynucleotides. Juodka *et al.*, 1991, demonstrated that the proteins of the nuclear DNP complexes are linked to DNA via phospho-triester bonds between hydroxy amino acid residues in peptides and internucleotide phosphates of the DNA. It seems likely that the cytoplasmic DNA sequences in the DNP complex are covalently linked to the proteins as well because of the following reasons: (i) the cytoplasmic DNP complexes exhibit the same stability of the DNA-protein interaction in the presence of high salt concentration, alkali, and detergents as the nuclear chromosomal DNP complexes, and (ii) the DNA associated proteins of the extrachromosomal DNP complex are related (or identical) to the proteins of the chromosomal DNP complex.

The chromosomal DNP complexes were separated from protein-free chromosomal DNA by filtration through nitrocellulose filters. DNA retained on the nitrocellulose filter represent a sub-set of DNA sequences of the genome and overlap with the DNA sequences found in preparations of the nuclear matrix (reviewed by Werner *et al.*, 1988). From these observations the nuclear DNP complexes are considered to be part of the nuclear matrix that is involved in the topological organization of the chromatin and in the tissue-specific regulation of gene expression. Noteworthy, the association of the nuclear polypeptides to DNA is DNA sequence-dependent, resulting in a non-random distribution of DNP complexes within the chromosomal DNA fiber (Werner and Neuer-Nitsche, 1989). The cytoplasmic DNP complexes, on the other hand, are exclusively found in tumor cells so far and do not seem to be constituents of the cytoplasm of normal cells (for review: Abken, 1995).

The physiologic function of the cytoplasmic DNP complexes in tumor cells is unresolved. Some of the cloned DNA sequences from Ehrlich ascites cells, e.g. EFC38 DNA, suppress the activity of the human c-fos promoter when assayed in a reporter construct transfected into human lymphoid cells. The EFC38 silencer activity seems to be promoter and cell specific and is directed to the promoter *in cis* as well as *in trans* via formation of a DNA-protein complex with the target promoter (B. Reifenrath-Biesel and H.A., manuscript in preparation). Preliminary experiments imply that the activity of the EFC38 DNA element to include unlimited proliferation of human lymphocytes *in vitro* is linked with its activity to form stable DNP complexes and to act as a silencer *in trans.* Formation of a stable DNA-protein complex by tight association of the DNA with the complex-forming proteins is an essential step in this process. In order to elucidate the function of the extrachromosomal DNA protein complexes with potential transcription regulatory activities, further experiments have to make clear the targets of the cytoplasmic DNP complexes within the chromosomal genome and the promoters and genes to be modulated. Protein-protein interactions of the homologous proteins of the cytoplasmic and the nuclear DNP complexes may result in association of DNP complexes to certain regions of the chromosomal DNA directing the extrachromosomal DNA elements to their nuclear targets.

We want to thank Dr. D. Werner (Heidelberg) for stimulating discussions and providing nuclear matrix associated DNA from Ehrlich tumor cells. Furthermore, we thank the Deutsche Forschungsgemeinschaft, Bonn, (Ab58/2-1), and the Boehringer Mannheim GmbH, Mannheim, for their financial support.

References

Abken, H. (1995) *The Cancer J. 8:*94-102.

Abken, H., Bützler, C. and Willecke, K. (1993) *Proc. Natl. Acad. Sci. USA 85:*468-472.

Abken, H. Hegger, R., Bützler, C. and Willecke, K. (1993) *Proc. Natl. Acad. Sci. USA 90:*6518-6522.

Abramova, Z. and Tsanev, R. (1987) *J. Mol Biol. 196:*437-440.

Bodnar, J.W., Jones, C.J., Coombs, D.H., Pearson, G. and Ward, D.C. (1983) *Mol. Cell Biol. 3:*1567-1579.

Capesius, I., Krauth, W. and Werner, D. (1980) *FEBS Lett. 110:*184-195.

Harlow, E. and Lane, D., (1988) *Antibodies: A Laboratory Manual.* Cold Spring Harbor, Cold Spring Harbor Laboratory Press.

Juodka, B., Pfütz, M. and Werner, D. (1991) *Nucleic Acids Res. 19:*6391-6398.

Krauth, W. and Werner, D. (1979) *Biochem. Biophys. Acta 564:*390-401.

Neuer, B. and Werner, D. (1985) *J. Mol. Biol. 181:*15-25.

Neuer-Nitsche, B. and Werner, B. (1987) *Biochem. Biophys. Res. Commun. 147:*335-339.

Plagens, U. (1978) *Chromosoma 68:*1-19.

Razin, S.V., Chernokhovostov, V.V., Cassetsky, W.S., Razina, M.V. and Geogiev, G.P. (1988) *Nucl. Acids Res. 16:*3617-3633.

Werner, D. and Neuer-Nitsche, B. (1989) *Nucl. Acids Res. 17:*6005-6015.

Werner, D., Rest, R. and Neuer-Nitsche, B. (1988) In: *Architecture of Eukaryotic Cells.* (G. Kahl, ed), VCH, Weinheim, pp. 449-459.

MINIREVIEWS

Physiol. Chem. Phys. & Med. NMR (1995) 27:331-338

PLANET: A Phage Library Analysis Expert Tool

Raphael Leplae and Anna Tramontano

Istituto di Ricerche di Biologia Molecolare (I.R.B.M.)
P. Angeletti Via Pontina Km 30.600 – I-00040 Pomezia, Rome, Italy

Abstract: In recent years random peptide libraries displayed on filamentous phage have been widely used and new ideas and techniques are continuously developing in the field (1-5). Notwithstanding this growing interest in the technique and in its promising results, and the enormous increase in usage and scope, very little effort has been devoted to the implementation of software able to handle and analyze the growing number of phage library-derived sequences.

In our laboratory, phage libraries are extensively used and peptide sequences are continuously produced, so that the need arose of creating a database (6) to collect all the experimental results in a format compatible with GCG sequence analysis packages (7). We present here the description of an XWindow-based software package named PLANET (Phage Library ANalysis Expert Tool) devoted to the maintenance and statistical analysis of the database.

Results

Peptide database

The database is composed of three parts: the first contains all non-redundant phage-derived nucleotide sequences, the second contains the deduced amino acid sequences derived from the entries in the first one, and the last includes all redundant nucleotide sequences. Each entry in the database contains information about the author's name, the experimental procedure, the amino acid or nucleotide sequence, etc. (Figure 1). The database currently contains more than 500 entries.

The program

PLANET was written in C using Xwindow libraries on a Silicon Graphics. This choice guarantees portability to several platforms and especially the possibility of having a user-friendly window and menu-based interface. This is particularly important since the program

```
Lib.: pVIII 9aa; Ab: MAb anti-VQGEESNDK (peptide from human interleukin 1
beta); Assay: dot blot and micropanning; Sel.: biopanning;
Au: Felici; 6-JUL-92
C: see J.Mol.Biol. (1991) 222, 301-310
C: P8il-17

Felici6  Length: 9  July  1, 1995 14:09  Type: P  Check: 3438  ..

   1 SNDGVWAIP
```

FIGURE 1. An example of an entry in the database.

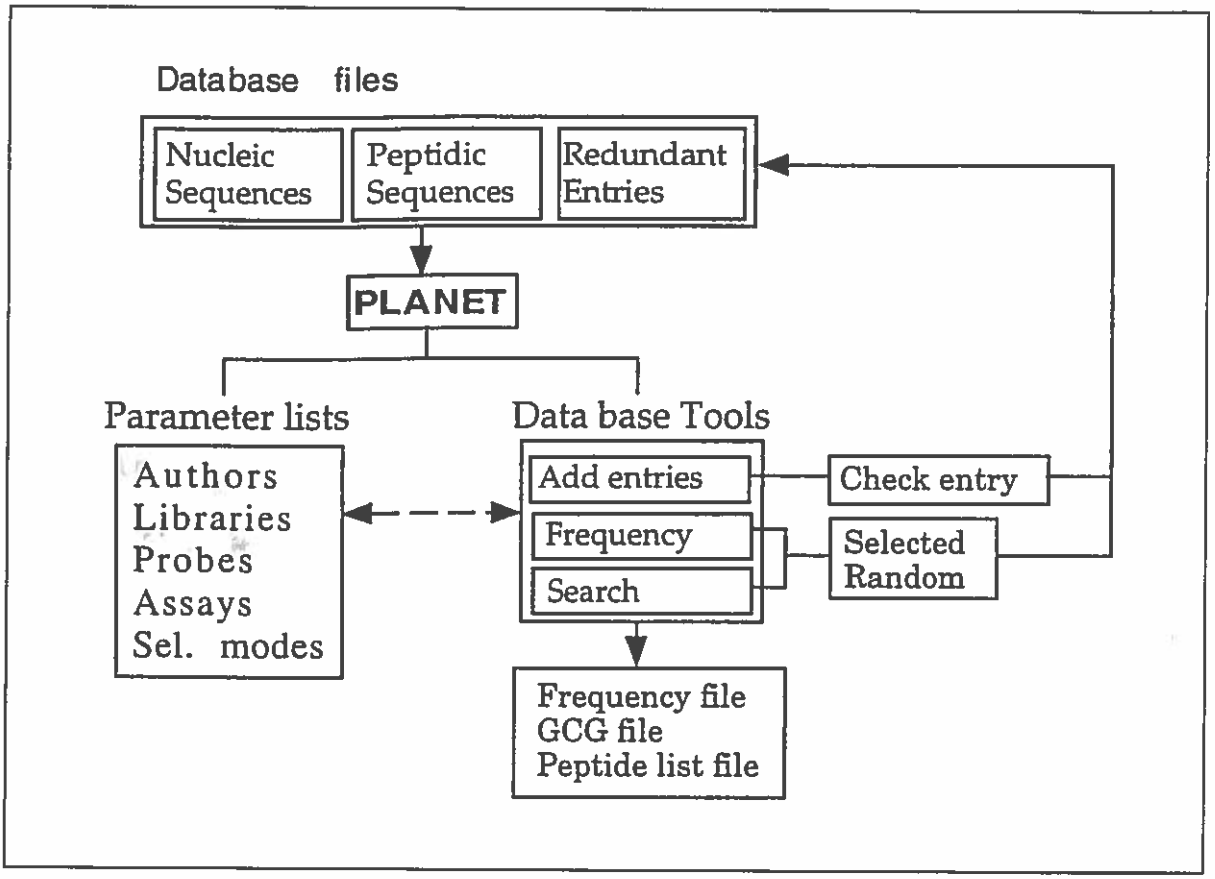


FIGURE 2. Schematic structure of PLANET.

should be easily accessible to experimentalists, both for introducing new data in the database and for analyzing them.

The schematic structure of PLANET is represented in Figure 2.

Database management

When the program is launched, the database is quickly pre-processed in order to retrieve all non-redundant entries. The displayed window appears as in Figure 3 and we call this the "PLANET window."

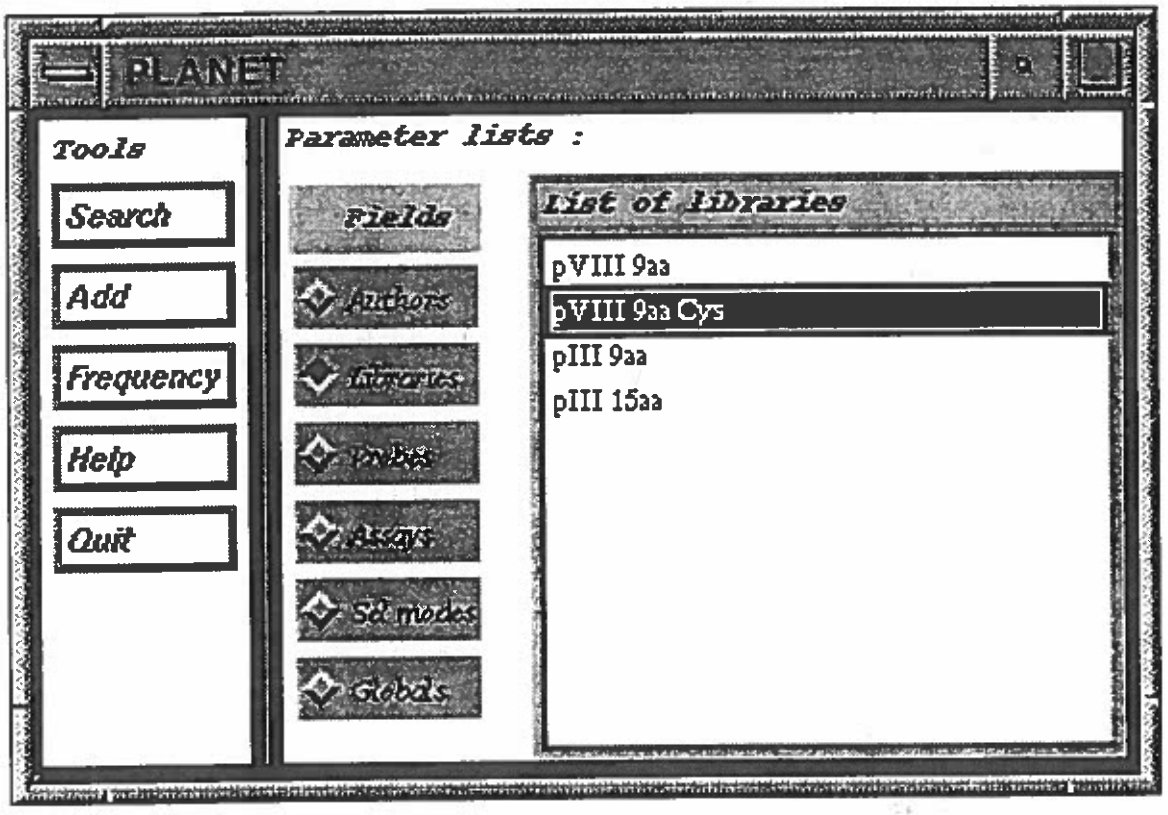


FIGURE 3. The PLANET window.

This window is divided into two parts. In the leftmost toggle menu entitled "tools" the user can select one of the Add, Search or Frequency options. The selection of each of these options causes the display of a window where the relevant fields can be filled. The "Search window", "Add window" and "Frequency window" are shown in Figures 4, 5 and 6, respectively and described below.

The rightmost part of the PLANET window entitled "Parameter lists" contains a list of fields which can be selected using toggle buttons and a list of values. Once a field is selected all the values that the field has assumed in the database are displayed. As we will see, this feature allows the user to enter the desired value by just double clicking on it, when any of the Search, Add or Frequency tools is selected.

Searching the database

The database created by PLANET is stored in GCG format, so that all the available sequence searching and analysis tools of this package can be directly used for this database.

There are however some special search features that are related to the special nature of this database which have been added to PLANET. They are activated by selecting the Search button in the PLANET window which causes the Search window to be displayed (Figure 4) for the selection of the fields to be searched.

Once a field is selected, for example "Libraries", the PLANET window automatically displays the list of libraries and double clicking on one of them will cause that value to be added to the query items for the search. Queries can be combined using logical operations and the search will start when the "Show result" option is selected. The resulting output is

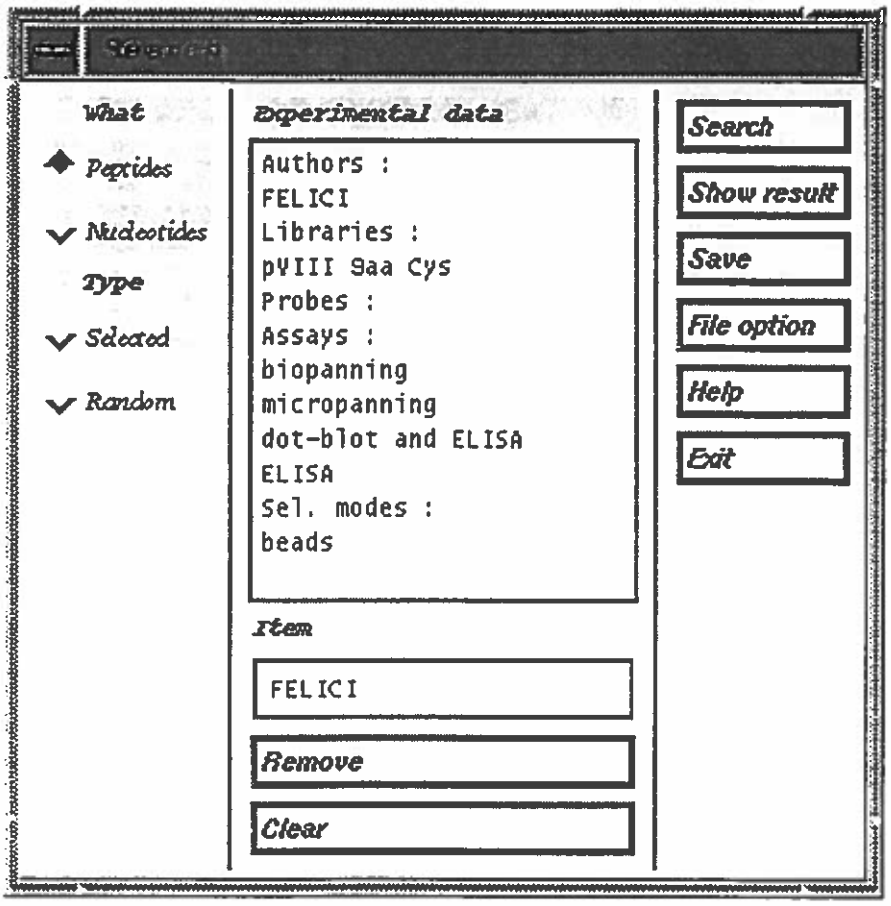


FIGURE 4. The Search Window.

displayed in a separate window (and can be saved to a disk file) in the same format as the database.

Adding entries into the database

The *Add* option allows the user to input new phage library derived sequences and related parameters into the database.

The option is designed to maximize efficiency and minimize errors. When selected, the "Add window" is displayed (Figure 5a). The user name is automatically used as the author of the experiment. The next field describes the library type. We use the convention according to which the first string is the phage protein used for display, either pIII or pVIII, the second is the length in amino acids of the random insert and the third is the (optional) string Cys used when the insert is flanked by two cysteine residue to favor a more constrained conformation of the peptide. The next field in the "Add window" is the name of the probe molecule, that is the protein used to select the phage library, as for example an antibody or the serum of a patient. The next two fields describe the assay used to check the positivity of the phage particles and the selection mode used, for example coating beads with the phage or biopanning. The last mandatory field is the nucleotide sequence itself.

At last, the user can invoke a text editor (by selecting the Edit comment button) to enter supplemental comments about the entry (Figure 5b).

With the exclusion of the sequence and comment fields, all input can be done by only

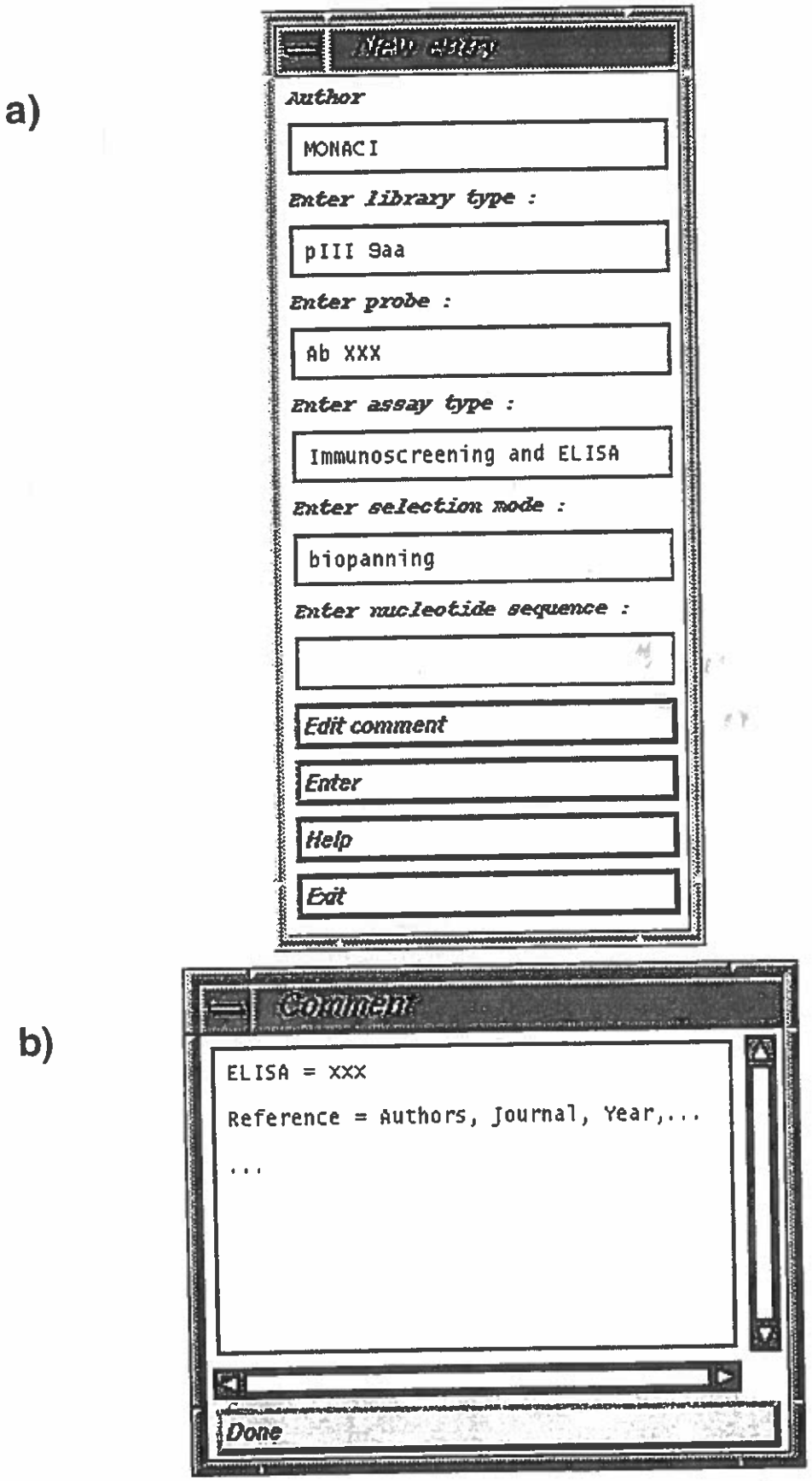
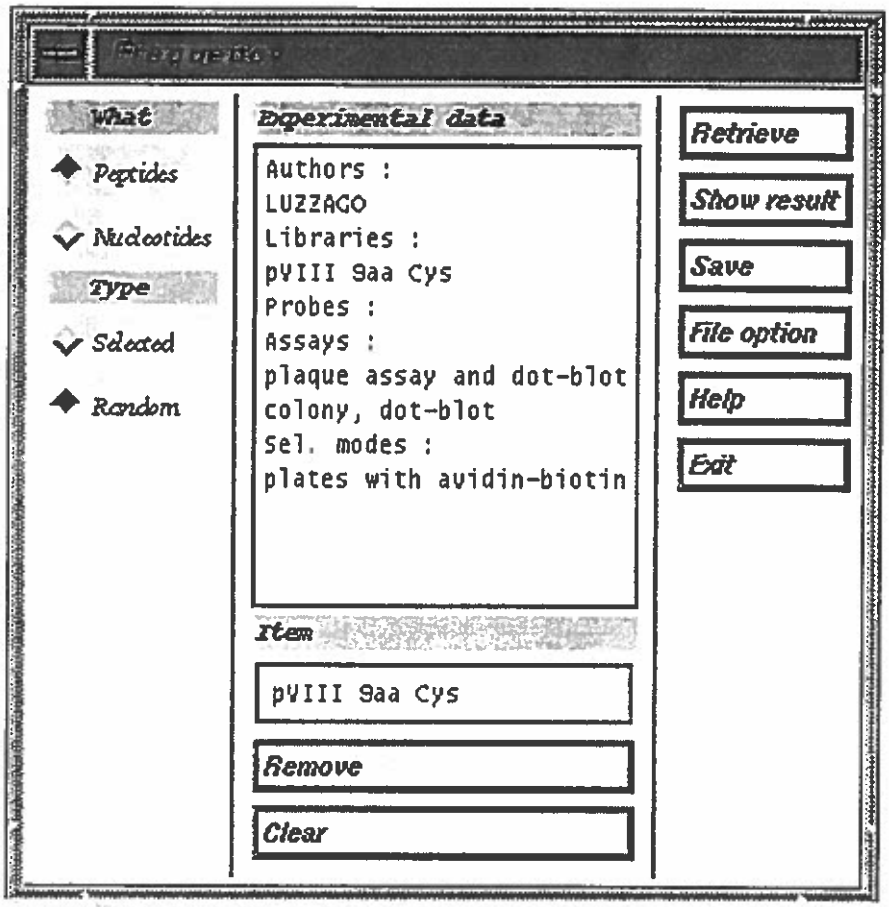


FIGURE 5. The Add window (a) and the Comment editing window (b).

a)



b)

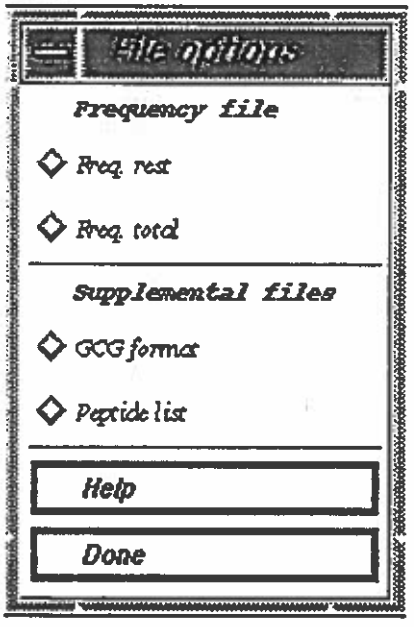


FIGURE 6. The Frequency window (a) and the file type menu (b).

using the mouse. As described before, when the cursor is positioned into one of the input fields, for example that named "Enter library type", the PLANET window will automatically select that field and consequently display all its existing values. Double clicking on one of these values will automatically fill the corresponding input field.

This possibility makes the input of new data faster, but what is more important, strongly reduces both the occurrence of typing errors and the usage of alternate names for the same value (for example two commonly used names for a given antibody being used by different authors). This is very relevant especially because it guarantees the completeness of the data retrieved according to the content of one of the entry fields.

When the new entry is completed and the user selects the Enter option, each field is checked for its correctness, the nucleotide sequence is translated into amino acids and compared with all other entries in the database to ascertain its non-redundancy. If a mandatory field is empty, or another type of error occurs, PLANET will prompt the user to complete or correct the entry. At the end of the checking step, PLANET will ask confirmation before saving the new entry.

The window for the last submitted entry is not automatically closed at this stage. Consequently, if a subsequent entry shares one or more fields with the previous one (for example because it was selected in the same experiment), only the different fields need to be modified before selecting the "Enter" option again.

Using the frequency retrieval tool

PLANET was designed to calculate residue frequencies of the peptides in the data base according to several criteria. The aim of this set of options is to answer questions such as: "Are proline or glycine residues more frequent in the selected sequences when the insert is flanked by cystine residues?" or "Is the charge distribution of the selected peptides correlated with with the type of selection experiment?" and so on.

The frequency tool allows the extraction of residue frequencies according to phage library type, probe used, assay types and selection modes in a separate or combined way. Analogously to what happens for the Search and Add options, PLANET will display the appropriate window and selection of one field will cause the PLANET window to show the list of its values, allowing double clicking to input data (Figure 6a).

When the user is satisfied with his/her selection of the fields, the "retrieve" option can be selected to obtain the requested frequencies. It is thus possible to obtain the frequency of the amino acid Proline in a non selected pIII library, or the frequency of charged amino acid in any library after selection with one or more monoclonal antibody.

The retrieved frequencies can be stored in one of three different formats (Figure 6b). A frequency file is a format compatible with several statistical analysis software (including Excel©). The user can select whether the file should only contain the retrieved frequencies or also the total residue frequency in the whole database and/or the difference between these two sets.

The second format contains all the peptide entries selected with PLANET in a GCG format.

The third and last option will produce a simple list of the retrieved peptides.

Conclusions

PLANET is the first menu-based program devoted to the management of data produced by random library screening,

We believe that the possibility of rapidly inputting and accessing data, by using any Xwindow compatible machine, will add to the ability of both retrieving data and to statistically analyze the whole phage-library derived database.

Our understanding of the potentials and limitations of random library screening is still limited, but a great wealth of data is already available and needs to be efficiently used and analyzed in order to gain insight into a number of characteristics of this system, as for example the bias introduced into the composition of the random peptides by the usage of a given library, the randomness of the library itself and so on.

We believe that the system described here represents a powerful tool to reach this goal and we plan to continue its development. The immediate steps will be the direct interfacing of the system with the GCG package and the addition of functions for direct statistical analysis.

We are grateful to all our colleagues at IRBM for many helpful discussions. Special thanks go to Drs. Armin Lahm, Franco Felici and to Professor Riccardo Cortese.

References

1. Scott, J.K. and Smith, G.P. (1990) *Science 249:*386-390.
2. Felici. F., Castagnoli, L., Musacchio, A., Jappelli, R. and Cesareni, G. (1991) *J. Mol. Biol. 222:*301-310.
3. Luzzago, A., Felici, F., Tramontano, A., Pessi, A. and Cortese, R. (1993) *Gene 128:*51-57.
4. Gallop, M., Barrett, R.W., Dower, W.J., S.P.A., F. and E.M., G. (1994) *J. Med. Chem. 37:*1233-1251.
5. Cortese, R., Monaci, P., Nicosia, A., Luzzago, A., Felici, F., Galfré, G., Pessi, A., Tramontano, A. and Sollazzo, M. (1995) *Curr. Opinion in Biotechnology 7:*73-80.
6. Tramontano, A., Pizzi, E., Felici, F., Luzzago, A., Nicosia, A. and Cortese, R. (1993) *Gene 128:*143-144.
7. Devereux. J., Haeberli, P. and Smithies, O. (1984) *Nucl. Acids Res. 12:*387-395.

Physiol. Chem. Phys. & Med. NMR (1995) 27:339-343

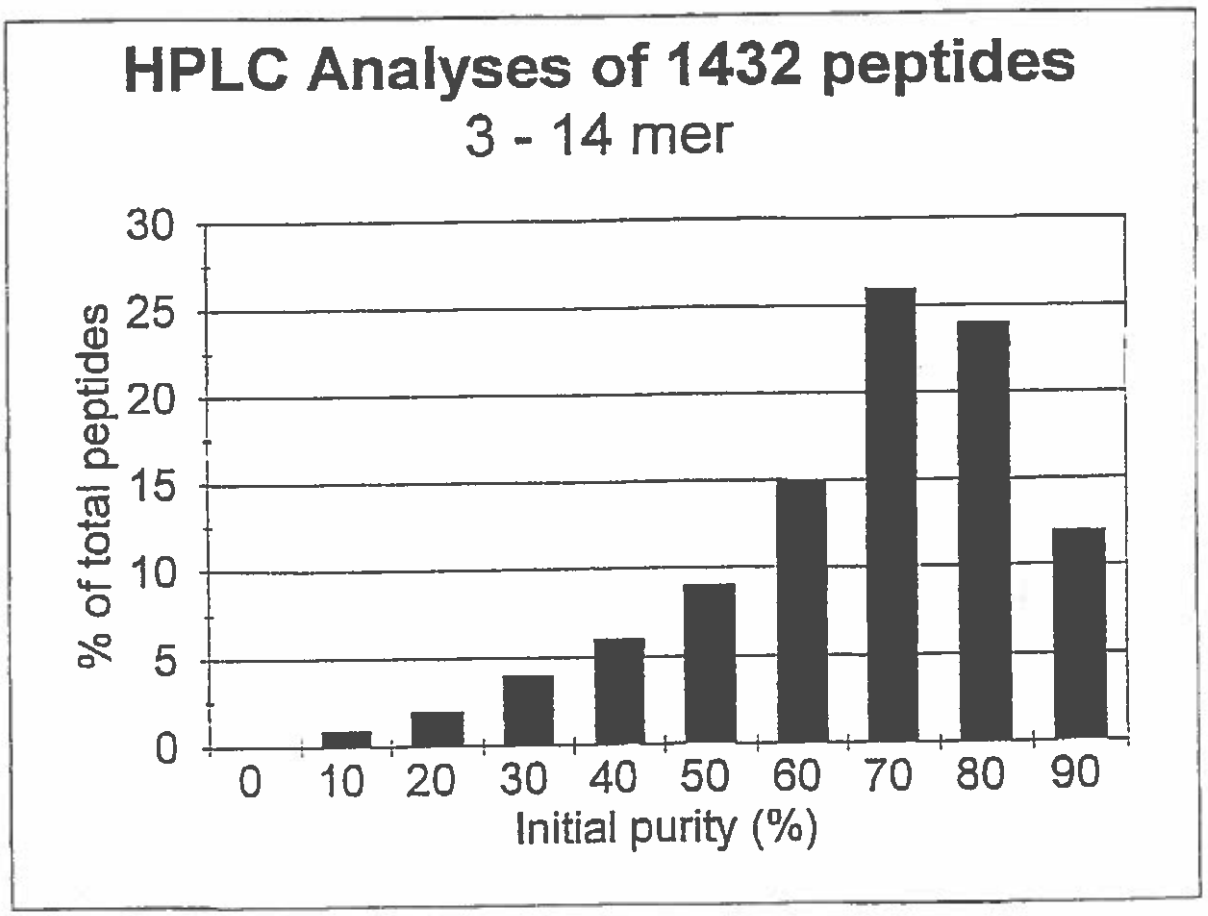
Multiple Synthesis Using the Multipin Method

Laurent Dapremont[1], Robert M. Valerio[2], Andrew M. Bray[2], Karen M. Stewart[2], Tom J. Mason[2], Alice W. Wang[2] and N. Joe Maeji[2]

[1]Chiron Mimotopes, 10 rue Chevreul, 92150 Suresnes, France and
[2]Chiron Mimotopes Pty. Ltd., 11 Duerdin Street, Clayton, Victoria, 3168, Australia

THE OPPORTUNITY to generate large arrays of compounds by directed assembly of simpler molecular building blocks, i.e., combinatorial synthesis, has enormous potential for the rapid synthesis of novel compounds in future drug discovery (1,2,3). This field covers many different synthetic methodologies, but whether peptides, oligonucleotides or small molecule organic compounds are being synthesized, and whether they are synthesized as mixtures or single compounds, the technology of multiple solid phase synthesis will play a dominant role. Multiple solid phase synthesis had its origins in the peptide arena where two methodologies for the synthesis of thousands of individual peptides were developed in the early 1980's. The first was the Multipin (4,5) system followed by the T-bag approach (6). Since that time, many other multiple synthesis methods have appeared, notably fully automated systems able to assemble up to 96 peptides at a time. But the original methods have also evolved and still maintain certain advantages over their more "modern" counterparts.

The multipin method was designed around modular principles. It was principally designed as an epitope mapping tool with individual pins arranged in an 8 × 12 format to complement the 96 well microtitre plate. Peptides were assembled on the tips of plastic pins, and by multiple handling of pin holders, thousands of individual peptides could be synthesized. The individual pin is composed of some rigid chemically stable plastic (normally polyethylene or polypropylene) on which a more mobile polymer is covalently bound (7). The latter is the resin. The pin itself can be considered its "container," and like any other container, it is not restricted to one particular type of resin. Polymers of very different physicochemical characteristics can be grafted onto the surface of pins and this opportunity for polymer selection allows an extra dimension of optimizing the surface for specific applications. For example, the surface used for epitope mapping, for enzyme inhibition



studies, and for large quantities of cleaved peptides are all different. In solid phase chemistry, one is dealing with a solvent/polymer matrix. Use of just one type of solid support can be likened to trying different organic reactions while restricted to only one solvent condition. There are also different size pins that allow synthesis of peptides from 40 micrograms to the 40 milligram range assuming an average 10-mer sequence. All pins versions can snap fit onto a common stem and the 96 pin format is still maintained even at the higher loadings.

Within our organization, there is the capability to simultaneously handle 32×96 peptides or a weekly output of approximately 3000 peptides assuming lengths in the 10- to 15-mer region. Figure 1 shows an overall indication of peptide purities for a sample of 1432 peptides under the 15-mer range. To be effective, multiple handling must not be restricted to compound assembly but encompass all steps of the synthesis process and beyond to testing. An example of such a concept is the diketopiperazine forming linker (8). Utilization of this linker allows simultaneous side chain deprotection and washing of hundreds to thousands of peptides in common baths. However, the linker cleaves in aqueous buffer at physiological pH to release peptides for immediate cellular assays. Another example is the use of electrospray MS linked to an autosampler to obtain peptide identity and purity as well as identity and relative abundance of all peptidic by-products in the sample. This data can be obtained within 3 mins per sample using an algorithm that matches the raw MS data with the predicted molecular ion resulting from possible side reactions that could arise during

synthesis (9). The algorithm analyzes the data and prints out the outcome with minimal human involvement. Multiple solid phase synthesis cannot simply be regarded as doing, in parallel, procedures that are suitable when handling a far smaller number of compounds. Organizational efficiency and computer support in all aspects of multiple synthesis and analysis are required.

The idea of generating mixtures of peptides from which optimum binding sequences could be identified by repetitive resynthesis of smaller and smaller mixtures was developed soon after the development of the pin concept (10). The original idea was based around the synthesis of every dipeptide within an octapeptide where all other positions were mixtures of the 20 naturally occurring amino acids. This library comprises a total of 400 pools with each pool having 64 million peptides. Since that time, many other libraries of different designs and mixture complexity have been developed by many different groups but there is basically two approaches to mixture generation, i.e., mixture coupling (10) and the split resin approach (11,12). The multipin methodology is incompatible with the split resin approach as the procedure involves small beaded particles (resins) being divided into a number of packets for optimized coupling of each individual amino acid. After coupling is completed, the resin packets are re-combined, mixed and then redivided for the next cycle of peptide assembly. Unlike mixture couplings where variable reactivities do lead to uneven amino acid distributions, split resin procedures result in equimolar distribution, in theory. However, this does not necessarily mean that the split resin approach is superior. Equimolar amino acid incorporation is necessary but insufficient to create a library with equilmolar distribution of its components. This is because the practicalities of synthesizing relatively large libraries mean that there is normally only a few resin beads for every member of a library. Such a situation can be described by a Poisson distribution (9). For example, synthesis of a 64 million member library using 64 million beads by the split resin approach results in 36% of the library not being synthesized at all. This is a theoretical analysis that does not take into consideration any of the practical aspects of synthesizing mixtures. Figure 2 shows the Poisson distribution and Monte Carlo simulation of a split resin library using 1 and 10 beads per peptide. With increasing bead numbers per compound, the distribution improves but then, the required quantity of resin can dramatically increase. How the library is generated, i.e., mixture coupling or split resin, pins or beads, number of beads used, total resin weight, type of resin, etc., affect the final composition of a library. Depending on synthesis conditions, mixture coupling procedures can generate as good or better libraries. For example, mixture coupling to one type of grafted pin gave an amino acid ratio of 1:1.85, least to most incorporated. Simplistically, a library comprising 4 mixture positions will have a concentration range of $1:1.85^4$, i.e., 1:11.6. While reality will give a far greater distribution range, the above discussions do indicate that library generation is a more complicated issue with no one methodology being superior to another.

An alternative to large mixture libraries is the generation of non-peptide libraries using a greater diversity of starting monomers than is available from the limited range of commercially available amino acids. Peptoids, i.e., N-substituted glycine polymers (13) is one example of a non-peptide polymer library. Using an alternating cycle of coupling bromoacetic acid followed by some amine, N-substituted glycine polymers can be quickly assembled using the large selection of commercially available amines (14). But, expanding the number of compounds in a library is not the only approach to increasing diversity. By computational methods, amines can be selected for generating biased libraries to a given

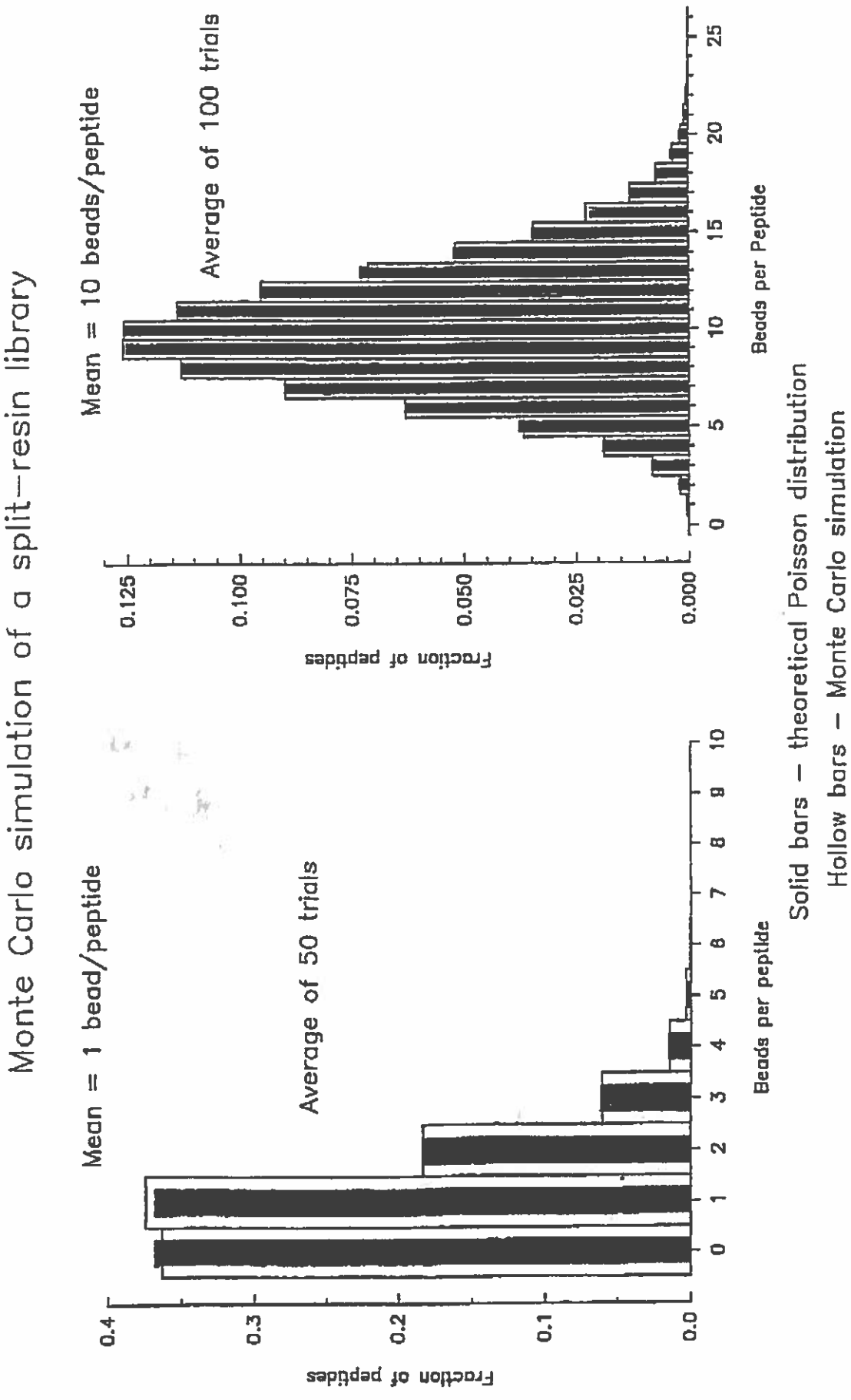

Monte Carlo simulation of a split–resin library
Mean = 1 bead/peptide
Average of 50 trials
Fraction of peptides
Beads per peptide
Mean = 10 beads/peptide
Average of 100 trials
Fraction of peptides
Beads per Peptide
Solid bars – theoretical Poisson distribution
Hollow bars – Monte Carlo simulation

FIGURE 2.

receptor or a family of receptors, or selection criteria could be biased for maximum diversity (15). Another non-peptide polymer is based on diaminopropionic acid (Dap). Here oligomers can be generated using commercially available carboxylic acids using conventional peptide chemistries. The multipin method has been used to assemble such non-peptide oligomer libraries as well as small molecule organic compounds such as benzodiazapines (16). The technique is suitable for many solid phase reactions and the future challenge is to progressively expand this repertoire of reactions in multiple solid phase organic chemistry.

References

1. Pavia, M.R., Sawyer, T.K. and Moos, W.H., Eds. (1993) *Bioorg. Med. Chem. Lett. 3:*381-475.
2. Gallop, M.A., Barrett, R.W., Dower, W.J., Fodor, S.P.A. and Gordon, E.M. (1994) *J. Med. Chem. 37:*1233-1251.
3. Gordon, E.M., Barrett, R.W., Dower, W.J., Fodor, S.P.A. and Gallop, M.A. (1994) *J. Med. Chem. 37:*1385-1400.
4. Geysen, H.M., Meloen, R.H. and Barteling, S.J. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81:*3998-4002.
5. Maeji, N.J., Bray, A.M., Valerio, R.M. and Wang, W. (1995) *Peptide Res. 8:*33-38.
6. Houghten, R.A. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82:*5131-5135.
7. Maeji, N.J., Valerio, R.M., Bray, A.M., Campbell, R.A. and Geysen, H.M. (1994) *Reactive Polymers 22:*203-212.
8. Bray, A.M., Maeji, N.J., Valerio, R.M., Campbell, R.A. and Geysen, H.M. (1991) *J. Org. Chem. 56:*6659-6666.
9. Mason, T.M., Bennell, P.S., Smart, S.S., Valerio, R.M., Bray, A.M., Maeji, N.J. and Geysen, H.M. (1995) In: *Peptides 1994*, (J.L.S. Maia, ed), ESCOM, The Netherlands, p. 94.
10. Geysen, H.M., Rodda, S.J. and Mason, T.J. (1986) *Mol. Immunology 23:*709-715.
11. Lam, K.S., Salmon, S.E., Hersh, E.M., Hruby, V.J., Kazmierski, W.M. and Knapp, R.J. (1991) *Nature 354:*82-84.
12. Houghton, R.A., Appel, J.R., Blondelle, S.E., Cuervo, J.H., Dooley, C.T. and Pinilla, C. (1992) *BioTechniques 13:*412-421.
13. Simon, R.J., Kania, R.S., Zuckerman, R.N., Huebner, V.D., Jewell, D.A., Banville, S., Ng, S., Wang, L., Rosenberg, S., Marlowe, C.K., Spellmeyer, D.C., Tan, R., Frankel, A.D., Santi, D.V., Cohen, F.E. and Bartlett, P.A. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89:*9367-9371.
14. Zuckermann, R.N., Kerr, J.M., Kent, S.B.H. and Moos, W.H. (1992) *J. Am. Chem. Soc. 114:*10646-10647.
15. Martin, E.J., Blaney, J.M., Siani, M.A., Spellmeyer, D.C., Wong, A.K. and Moos, W.H. (1995) *J. Med. Chem. 38:*1431-1436.
16. Bunin, B.A., Plunkett, M.J. and Ellman, J.E. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91:*4708-4712.

Physiol. Chem. Phys. & Med. NMR (1995) 27:345-349

Acidic Chromatin Peptides. Molecular Models and Biological Activity

G. Lugaro[1], L. De Angelis[2], F. Giavarini[2], V. Marsili, L. Mancinelli, E. Castigli and G.L. Gianfranceschi

[1]Istituto di Chimica degli Ormoni, C.N.R., Milano, Italy;
[2]Istituto di Scienze Farmacologiche, Università di Milano, Italy;
Dipartimento di Biologia Cellulare e Molecolare, Università di Perugia, Perugia, Italy

OVER MANY YEARS we have reported the isolation and characterization of low molecular weight peptides probably involved in the control of transcription. These peptides have been found both in prokaryotic and eukaryotic cells.

In the eukaryotics, the peptides have been found free in biological fluids such as human and calf blood or in seminal plasma, while inside the cell, in the nucleus, they are bound to chromatin DNA. The tissues most utilized for the extraction of the peptides are spermatozoa, calf thymus, trout testis, pea buds and wheat germ. With regard to the prokaryotic cells, we have found similar peptides associated to DNA of *E. coli* and λ phage (1,2).

The isolated peptides are purified by ion exchange chromatography, gel filtration and HPLC. However at the end of the purification processes the peptide fraction still appears to be composed of a family of peptides with similar amino acid composition and structure. Another difficulty for the progress of the purification procedure is the small amount of purified material obtained.

In any case despite the difficulty represented by the presence of a family of similar peptides and the small amount of available purified peptide, some structural characteristics have been demonstrated: 1) a molecular weight of about 1000; 2) the blocked N-terminal probably constituted by pyroglutamic acid; 3) the predominant presence of glutamic acid (between 2 and 6 residues), aspartic acid (1-3 residues), serine (1), glycine (1) and alanine(1). 4) Moreover they are phosphorylated at the level of serine.

The biological activity of the isolated peptides appears to be related to the control of transcription *in vitro* in reconstituted systems and in cellular systems (Table I) (3). The addition of the peptide fraction strongly inhibits at low concentration the DNA or chromatin transcription *in vitro* carried out by prokaryotic or eukaryotic RNA polymerases. In regard to the experiments performed on cellular systems, we demonstrated that the peptides inhibit

TABLE I.

BIOCHEMICAL ACTIVITY OF NATIVE PEPTIDES

CELL-FREE SYSTEMS	CELL SYSTEMS
-DNA transcription	-RNA synthesis:
-Chromatin transcription	human leukemia leukocytes
-RNA synthesis:	lymphocytes stimulated by PHA
isolated nuclei from	L1210 leukemia cells
hepatocytes and PC-12 cells	-Globin mRNA transcription:
	Friend's leukemia cells
	-Growth and differentiation:
	HL60 leukemia cells

the RNA synthesis in human leukemic leucocytes and in lymphocytes stimulated by phytohemagglutinin. We have also observed that the transcription of the globin messenger RNA induced by dimethylsulfoxide in Friend's leukemia cells is inhibited by the peptide fraction. Moreover the peptides we isolated are able to control the RNA transcription in L1210 leukemia cells (3). On the other hand we demonstrated that the level of the low molecular weight peptides bound to chromatin is strongly decreased in cancer cells as compared with normal cells (Figure 1). Following these results, more recently we tried to obtain more information about the peptide structure by means of mass spectrometry analysis. The mass spectrometry analysis can give good information on the possible peptide structure, also in a mixture of peptide sequences, because we can obtain the exact molecular weight of the peptides and also the molecular weight of the breakdown products. If the sample subjected to mass spectrometry is composed almost of only one peptide structure, especially for low molecular weight peptides, it is sometimes possible to completely design the structure (Figure 2). For a sample containing a mixture of peptides, also at higher molecular weight, the design of the sequences is more complicated, however some information may be obtained and it is sometimes possible to try to design a molecular model.

Table II shows the sequences of a series of peptides designed on the basis of combined information obtained from biochemical analysis and mass spectrometry analysis. Some of these peptides have been synthesized and we have tested their biological activity in cell-free and in cellular systems.

The biological activity of the synthetic peptide has been compared with the biological activity of the native peptides. The transcription reaction *in vitro* shows a dose-dependent strong inhibition by the peptide fraction purified from wheat germ chromatin (5). The

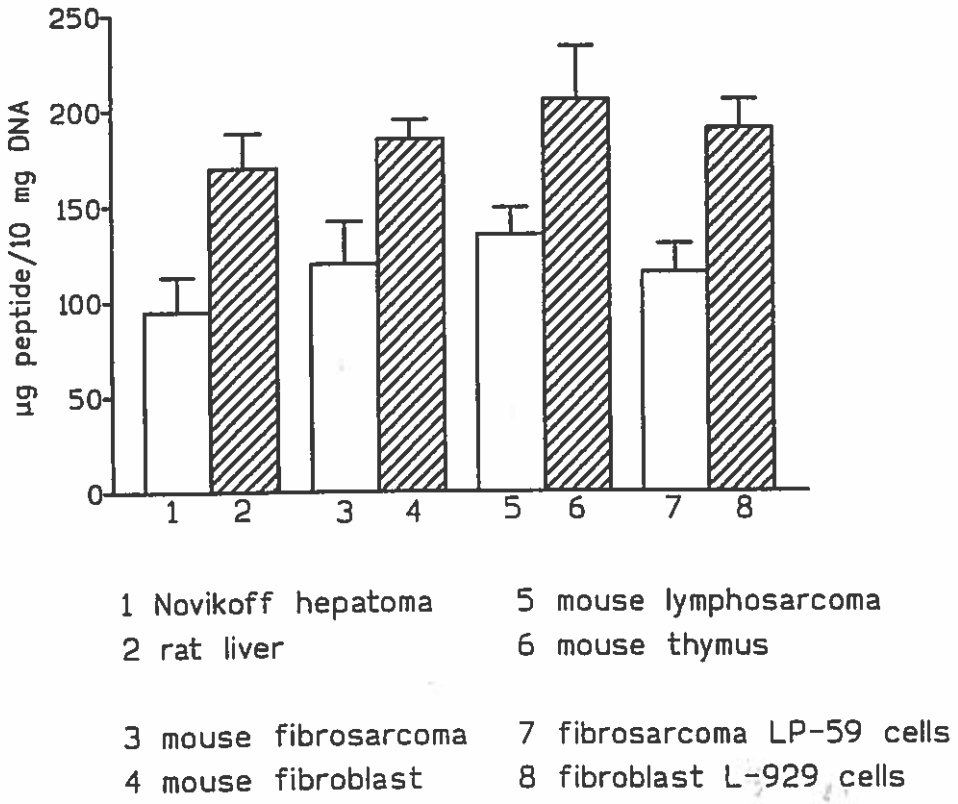


FIGURE 1. Quantitative evaluation of active low molecular weight peptides extracted from normal and cancer cells DNA (µg peptide/10mg DNA).

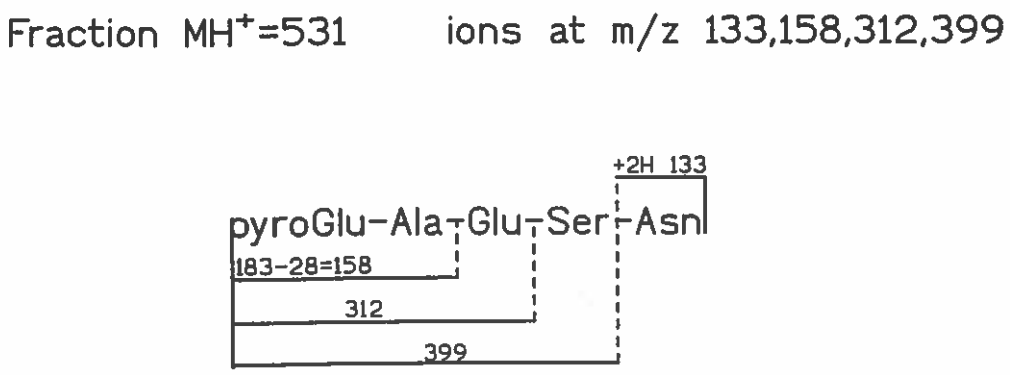


FIGURE 2. Schematic representation of the structure model designed for a pentapeptide pyroGlu-Ala-Glu-Ser-Asn (MH^+531) through the evaluation of the ions 133, 158, 312, 399 obtained from FIB^+ mass spectrum.

synthetic peptides have been tested on transcription utilizing different templates and RNA polymerases. The synthetic peptides in the unphosphorylated form do not modify the transcription rate. On the contrary the synthetic peptides previously phosphorylated *in vitro* by protein kinase CKII, cause a slight stimulation at very low concentration (<0.4 ng/µl) and a strong progressive inhibition at higher concentration (6). So in the DNA transcription performed *in vitro* in reconstituted systems, the effects obtained with the synthetic peptides are very similar to those shown by the native peptides.

TABLE II.

SYNTHETIC PEPTIDES

1) PYROGLU-ALA-GLU-SER-ASN
2) PYROGLU-ALA-GLY-GLU-SER-GLU-ASP
3) PYROGLU-ALA-GLY-GLU-GLU-SER-ASN
4) PYROGLU-ALA-GLY-GLU-GLU-GLU-GLU-SER-ASN
5) PYROGLU-ALA-GLY-GLU-ASP-SER-ASP-GLU-GLU-ASN
6) PYROGLU-ASP-ASP-SER-ASP-GLU-GLU-ASN
7) PYROGLU-VAL-ALA-ASP-SER-ASP-GLN-ASN
8) PYROGLU-VAL-ALA-ASP-THR-GLU-SER-GLU-PRO-ASN

Another model we use for testing the peptide activity is the transcription in isolated nuclei. The transcription in nuclei is interesting because it represents a more controlled system of transcription and consequently it appears closer to the physiological conditions of RNA synthesis. However the data available at the present time do not allow us to compare the activity of native peptides with that of synthetic peptides. In fact native peptides isolated from seminal plasma have been tested on nuclei isolated from rat hepatocytes and the effect is represented by a dose-dependent strong inhibition (Table III). Vice versa, the synthetic peptides have been tested in our laboratory on nuclei isolated from PC12 cells. In this system the peptides have been utilized in the unphosphorylated form by taking into account that the nuclei contain the protein kinase CKII and consequently the peptide should be subjected to endogenous phosphorylation. The results obtained show that the synthetic peptides are able to control the RNA synthesis in nuclei mostly by a stimulation of the RNA polymerase II activity but the complete understanding of the results is more complicated because the effect probably depends on several factors (7).

Concerning the activity at the cellular level, native and synthetic peptides are tested on the growth of HL60 cells, a human promyelocytic leukemia. The results show a strong inhibition caused on the HL60 cell growth by the peptide fraction isolated from seminal plasma. A very similar effect has been observed also with the peptides purified from the chromatin of pea buds or wheat germ (data not shown). The synthetic octapeptide pyroGlu-Asp-Asp-Ser-Asp-Glu-Glu-Asn is the one most studied in this system.

The effect observed on the growth of HL60 cells is represented by a slight inhibition reaching a maximum percentage value of 20–25%. This effect becomes much more evident if the peptide is added to permeabilized cells. In HL60 cells permeabilized with the Trans-Port Permeabilization kit from GIBCO BRL, the inhibition reaches about 50% after 6 days of incubation.

Moreover we have observed that the octapeptide causes a remarkable inhibition of growth rate in HL60 cells induced by 1% DMSO; the peptide at μM concentration causes a decrease of growth rate of about 40-45%. It is also interesting that the decrease of the proliferation rate is probably due to a parallel stimulus of the differentiation process of HL60

TABLE III.

CONTROL			ca. 10^4 cpm (incorporation of ^{3}H-UMP)
PEPTIDE	2	µg	82.6 % of inhibition
	1	µg	63.1 % "
	0.5	µg	45.5 % "
	0.25	µg	32.2 % "

Percent inhibition of RNA synthesis in rat epatocytes by different amounts of native peptides from seminal plasma.

cells. In fact it is evident that the peptide at the same concentration causes a significant stimulus of cell differentiation (8).

On the basis of these results our opinion is that the synthetic octapeptide is potentially able to control the cell proliferation of these leukemia cells but the activity is strongly quenched by its low ability to pass through the cell membrane.

In fact the effect is more evident in permeabilized cells and in cells grown in the presence of DMSO; the properties of DMSO as penetrant carrier have been reported by many authors.

In conclusion, we think that the synthetic peptides we designed have a structure very similar to that of native peptides and this is confirmed by the similar activity observed in cell free systems. In cell systems the activity of the synthetic peptides appears also potentially similar to that of native peptides but the native peptides probably pass more easily through the cell membrane.

Our researchers have work in progress to modify the structure of the synthetic peptides, adding at the N-terminal a hydrophobic tail to facilitate their internalization.

References

1. Gianfranceschi, G.L., Barra, D., Bossa, F., Coderoni, S., Paparelli, M., Vananzi, F., Cicconi, F. and Amici, D. (1982) *Biochim. Biophys. Acta 699:*138-148.
2. Lugaro, G., Campagnari, F., Moretti, R. and Casellato, M.M. (1988) *Biochim. Biophys. Acta 950:*420-428.
3. Amici, D., Rossi, G., Cioé, L., Matarese, G.P., Dolei, A., Guglielmi, L. and Gianfranceschi, G.L. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74:*3869-3873.
4. Gianfranceschi, G.L., Amici, D. and Guglielmi, L. (1976) *Nature 262:*622-623.
5. Mancinelli, L., Castigli, E., Franceschini, M., Gianfranceschi, G.L., Bramucci, M., Miano, A. and Amici, D. (1992) *Physiol. Chem. Phys. & Med. NMR 24:*97-107.
6. Angiolillo, A., Desgro, A., Marsili, V., Panara, F. and Gianfranceschi, G.L. (1993) *Experientia 49:*902-905.
7. Castigli, E., Mancinelli, L., Mariggió, M.A. and Gianfranceschi, G.L. (1993) *Am. J. Physiol. 265 (Cell Physiol. 34):*C1220-1223.
8. Marsili, V., Angiolillo, A. and Gianfranceschi, G.L. (1995) *Cell. Mol. Biol. 41:*515-523.

Physiol. Chem. Phys. & Med. NMR (1995) 27:351-353

Biologically Active Pyroglutamyl N-Terminal Oligopeptides: Parts of Larger Molecules?

[1]Kjell Elgjo, [2]Karl L. Reichelt, [2]Wenche H. Reichelt

[1]Institute of Pathology and [2]Pediatric Research Institute, The National Hospital (Rikshospitalet), N-0027 Oslo, Norway

Abstract: In 1984 we identified and characterized a growth-inhibiting pentapeptide, pyroGlu-Glu-Asp-Ser-GlyOH, [EPP] from mouse epidermis. Later, other pyroGlu N-terminal oligopeptides have been isolated and characterized from liver and mouse intestine. The three pyroGlu-terminal mitosis inhibitory peptides are structurally similar and have several biological properties in common.

A number of questions remain, however, to be answered, e.g., a) Are the peptides part of larger molecules; b) Do they bind to specific receptors on the target cells; c) How are they related to other growth-modulating factors, and c) Are they coded for by genes that are related to known growth regulating proto-oncogenes, especially to growth suppressing genes.

To search for soluble parts of possible receptors, or carrier molecules, in water extracts of mouse epidermis we have used affinity columns coated with EPP with either the N-terminal end or the carboxy-end free. Both types of column bind a 70 kD protein. The protein bound to the column with a free N-terminal end splits into two small components under reducing conditions. To look for larger molecules of which EPP could be a fragment, we have used western blotting techniques and a polyclonal rabbit antiserum against EPP. Preliminary experiments have indicated that two different molecules bind to the antiserum.

OBSERVATIONS OF PATTERNS of cell proliferation in regenerating tissues have led us and others to the conclusion that growth regulation in general is based on a negative feedback principle. Almost 30 years ago it was shown that water extracts of skin or epidermis would reversibly inhibit cell division in the epidermis *in vivo* and *in vitro*, and that the effect seemed to be tissue-specific and species nonspecific (Bullough *et al.*, 1967). The extracts thus contained one or more factors that could act as signal(s) in a negative feedback control of epidermal growth. In 1984 we finally isolated and characterized a pentapeptide (pyroGlu-Glu-Asp-Ser-GlyOH) (EPP) and a dipeptide (pyroGlu-GlyOH) that had properties that made them likely as signal substances in a growth-regulating system based on a

negative feedback principle (Elgjo and Reichelt, 1984; Elgjo *et al.*, 1986a; Reichelt *et al.*, 1987; Elgjo and Reichelt, 1988).

At about the same time, Paukovits and Laerum identified a structurally similar pentapeptide that reversibly inhibited granulo-monocytopoiesis (Paukovits and Laerum, 1982). Some years later, our group isolated similar mitosis inhibitors from mouse intestine and from the liver (Skraastad *et al.*, 1988; Paulsen *et al.*, 1987). At the present time, we thus have 4 inhibitors with a similar biochemical structure and with biological properties: 1) pyroglutamate ring at N-terminal end, resulting in solubility in both water and organic solvents; 2) reversible inhibition of cell flux at the G_1-S and G_2-M transitions; 3) curvilinear ("bell-shaped") dose-response relationship and optimal effect at picomol doses/concentrations; 4) long-lasting effect of a single treatment (> 24 h); 5) refractory period of several hours after the initial treatment; 6) effect on transformed or malignant cells derived from the organ/tissue in which the respective peptides were found; 7) relative tissue specificity/preference.

The pyroGlu-ring and the dominance of a certain set of amino acids make these peptides very similar to those described by Italian research workers (Amici *et al.*, 1985; Gianfranceschi *et al.*, 1989; Felici *et al.*, 1991; Angiolillo *et al.*, 1993a; Angiolillo *et al.*, 1993b), and that were extracted from DNA. The latter peptides bind to DNA in their phosphorylated form, and recently Bramucci *et al.* (1992) showed that EPP is easily phosphorylated by protein kinase II.

The biological effects of EPP *in vivo* have been examined in several experiments (Elgjo and Reichelt, 1988; Elgjo *et al.*, 1986b; Elgjo and Reichelt, 1991) but several crucial questions remain to be answered: a) Do the peptides have specific receptors and, do the putative receptors belong to known receptor families; b) Are the peptides active fragments of larger molecules, and c) Which intracellular pathways are involved, if any? There is a partial answer to the last question. Experiments performed with EPP and mouse epidermis (Elgjo and Reichelt, 1994), and with EAT cells *in vitro* (Antokhin *et al.*, 1991) have demonstrated that blocking of β-receptors neutralizes the inhibitory effect on cell flux at the G_2-M transition but has no effect on cell flux at the G_1-S transition.

In order to search for possible surface receptors we have used affinity columns coated with synthetic EPP. The peptide was either bound to the column at the N-terminal (ECH Sepharose 4B gel) or the C-terminal end (EAH Sepharose gel) of the peptide. In order to make possible an attachment at the N-terminal end the pyroGlu-ring was opened. Linking to the gel was achieved by using carbodiimide to form a peptide-like binding. When supernatants of water extracts of mouse epidermis were run through the two types of columns a protein was bound which on a SDS-PAGE gel gave a component with a molecular weight of about 70 kD. When the protein obtained from the column with a free pyroGlu-end was treated with mercaptoethanol before analysis on a SDS-PAGE gel, an additional protein with molecular weight of about 50 kD was identified. The 70 kD protein could thus consist of two different subunits joined by a S-S bond, one of the subunits having a molecular weight of 50 kD, and the other being smaller. At the present time, we do not know whether the two 70 kD proteins obtained from the two different columns are identical, or two different proteins with similar molecular weights. Also, we do not know whether the substances that bind to the affinity columns are specific for the epidermis; whether they are soluble parts of receptors or, whether they are molecules of which EPP is a fragment.

To examine whether EPP is a fragment of a larger molecule, we needed an antibody against EPP. First, we made a monoclonal antibody that neutralized the inhibitory effect of

EPP *in vivo*. However, this antibody reacted even with other substances having an N-terminal pyroGlu-ring and was therefore of little practical value. To circumvent this problem we used a custom synthesized EPP with an opened pyroGlu-ring. The N-terminal end was attached to BSA and the complex used to immunize rabbits. In this way we obtained polyclonal antibody that neutralized the inhibitory effect of EPP when tested in mice, and that was detectable at a 1/2000 dilution in ELISA tests.

In preliminary experiments the polyclonal antibody was bound reproducibly to two different components in mouse epidermis dissolved in SDS and urea (Frank Brosstad, personal comm.). At the present time we have not yet obtained enough material for further analysis of the two components. It is thus too early to tell whether the components obtained by means of the antibody are related to those identified by means of affinity columns coated with EPP.

One of the main reasons why it is important to find out whether EPP and the other peptides are fragments of larger molecules is that a pentapeptide, not to mention a tripeptide, is too small for experiments aimed at identifying a gene, or genes. Only by identifying their gene(s) would we know whether the four identified mitosis-inhibitory peptides have any relationship with other known growth modulators, hormones, or signal substances such as neurotransmitters.

References

Antokhin, A.I., Popova N.Y., Gembitsky, D.S. and Romanov, Y.A. (1991) *Bulletin Exp. Biol. Med. 112:*1037-1039 (translated and published in USA).

Angiolillo, A., Desgro, A., Marsili, V., Panara, F. and Gianfranceschi, G.L. (1993a) *Experientia 49:*902-905.

Angiolillo, A., Bramucci, M., Marsili, V., Panara, F., Miano, A., Amici, D. and Gianfranceschi, G.L. (1993b) *Mol. Cell. Biochem. 125:*65-72.

Bramucci, M., Miano, A. and Amici, D. (1992) *Biochem. Biophys. Res. Comm. 183:*474-480.

Bullough, W.S., Laurence, E.B., Iversen O.H. and Elgjo, K. (1967) *Nature 214:*578-580.

Elgjo, K. and Reichelt, K.L. (1984) *Cell. Biol. Int. Rep. 8:*379-382.

Elgjo, K. and Reichelt, K.L. (1988) *Ann. NY Acad. Sci. 548:*197-203.

Elgjo, K. and Reichelt, K.L. (1991) *Arch. B. Cell. Path. 60:*161-164.

Elgjo, K. and Reichelt, K.L. (1994) *Epith. Cell. Biol. 3:*32-37.

Elgjo, K., Reichelt, K.L., Edminson, P. and Moen, E. (1986a) In: *Biological Regulation of Cell proliferation,* (F. Baserga, P. Foa, D. Metcalf and E.E. Polli, eds), Raven Press, New York, pp. 259-265.

Elgjo, K., Reichelt, K.L., Hennings, H., Michael, D. and Yuspa, S.H. (1986b) *J. Invest Dermatol. 87:*555-558.

Felici, F., Cardellini, E., Miano, A., Bramucci, M., Piccinini, G., Amici, D. and Gianfranceschi, G.L. (1991) *Mol. Biol. 15:*9-18.

Gianfranceschi, G.L., Cardellini, E., Piccinini, G., Bramucci, M., Miano, A., Maccari, E., Amici, D. and Durban, E. (1989) *Physiol. Chem. Phys. & Med. NMR 21:*289-294.

Paukovits, W.R. and Laerum, O.D. (1982) *Z. Naturforschung C. 37:*1297-1300.

Paulsen, J.E., Reichelt, K.L. and Petersen, A. (1987) *Virchows Arch. Cell. Path. 54:*152-154.

Reichelt, K.L., Elgjo, K. and Edminson, P.D. (1987) *Biochem. Biophys. Res. Comm. 146:*1493-1501.

Skraastad, Ö., Fossli, T., Edminson, P. and Reichelt, K.L. (1988) *Epithelia 1:*107-119.

Physiol. Chem. Phys. & Med. NMR (1995) 27:355-361

Acellular Pertussis Vaccine Composed of Genetically Inactivated Pertussis Toxin

Samuele Peppoloni, Mariagrazia Pizza, Maria Teresa De Magistris, Antonella Bartoloni and Rino Rappuoli

IRIS Research Institute, Biocine Spa, Via Fiorentina 1, 53100 Siena, Italy

Abstract: Whooping cough, an acute respiratory disease affecting over sixty million infants, can be prevented by vaccination. The vaccine currently used, composed of killed bacterial cells, however, has been associated with many side effects. An improved vaccine against the disease should contain pertussis toxin (PT), a major virulent factor of *Bordetella pertussis (B. pertussis)*. In order to be included in the vaccine, PT needs to be detoxified and the chemical methods used so far are not completely satisfactory, since they give a product with reduced immunogenicity and possible residual toxicity. To avoid this problem, we have used recombinant DNA technologies to clone the PT gene, express it in bacteria, map the B and T cell epitopes of the molecule and identify the amino acids that are important for the enzymatic activity and toxicity. Based on this information, the gene coding for PT was mutated to produce an inactive protein. This genetically modified PT was non toxic, highly immunogenic and able to protect mice from intracerebral challenge with virulent *B. pertussis*. The mutant was included as a main component of an acellular pertussis vaccine which has been shown in numerous clinical trials to be more safe and immunogenic than the old cellular vaccine.

WHOOPING COUGH is an acute respiratory disease affecting over 60 million infants and is responsible for approximately half a million deaths each year (1). The disease is caused by *B. pertussis*, a Gram-negative bacterium, that is transmitted by aerosol from contacts with the disease. Following infection, *B. pertussis* adheres specifically to the cilia of the upper respiratory tract, where it multiplies and releases several toxins that cause both local and systemic damages. Locally, the bacteria damages the respiratory epithelium and causes the loss of the cilia. The resulting clinical disease is characterized by long lasting paroxysmal cough, accompanied by whoops, vomiting, cyanosis and apnea. The most common complications are pneumonia, seizure, encephalopathy and death (2).

The vaccine currently used was developed in the 1940s and is made by whole bacteria killed and stabilized with formaldehyde, heated at 56ºC for 30 minutes to inactivate a

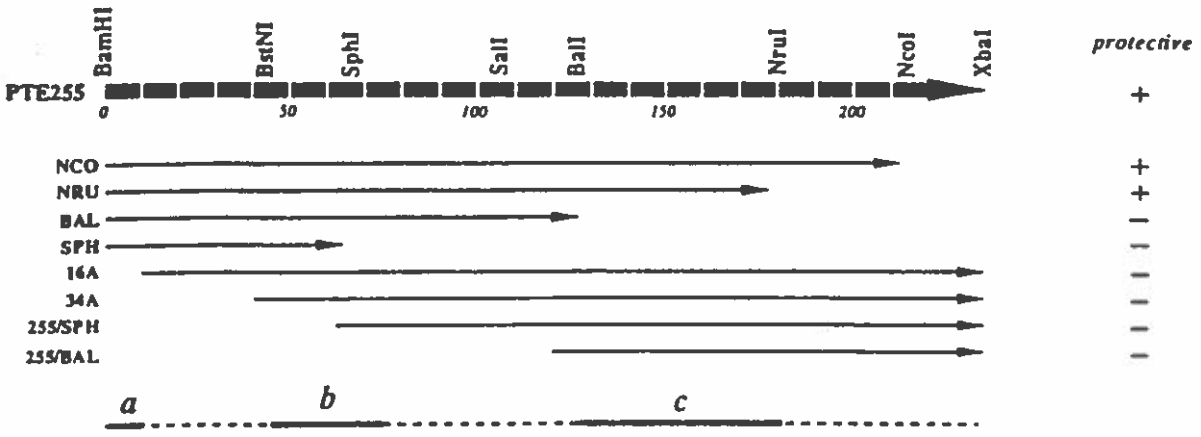


FIGURE 1. Map of S1 subunit and of the deletion mutants tested in this study. On the right, is shown the immunoreactivity (+ or -) of the protective monoclonal antibodies. The three regions (a, b and c) required for the binding are indicated at the bottom of the figure, whereas on the top are reported the restriction sites used to produce these mutants.

heat-labile toxin (3). Although effective in preventing the disease, this vaccine has been associated with mild reactions, such as redness, swelling and fever that occur in 20-70% of the vaccinees. In addition to these mild reactions severe side effects, such as anaphylaxis, brain damage and death have been associated in rare cases (3 to 9 cases per million doses) with pertussis vaccination (4).

As a result, pressure has built up to develop safer acellular vaccines against whooping cough (5). PT, a major virulence factor of *B. pertussis*, either alone or combined with other antigens, is the main component of all acellular vaccines so far developed. A large clinical trial carried out in Sweden has shown that chemically detoxified PT can prevent severe whooping cough with an efficacy equal to that of the cellular vaccine (6).

Unfortunately, the chemical treatments used to detoxify PT are not fully satisfactory, because they give a product with reduced immunogenicity, which in some cases has been shown to revert to toxicity (7).

Complete and stable detoxification of PT is therefore mandatory, since the severe complications of the cellular vaccine observed in vaccinees may be due to minute traces of residual active toxin. PT is a complex bacterial protein composed of five non-covalently linked subunits named S1 through S5 according to their electrophoretic mobility (8). S1 is an enzyme which has a toxic effect upon eukaryotic cells by ADP-ribosylating their GTP-binding proteins. Subunits S2, S3, S4 and S5, present in a 1:1:2:1 ratio, bind the receptors on the surface of eukaryotic cells and facilitate the translocation of the S1 subunit across the cellular membrane, so that it can reach the target proteins. The genes coding for the five subunits of PT were cloned and sequenced in our laboratory (9), as well as in the laboratory of Jerry Keith at the Rocky Mountains Laboratory, in Montana (10). The five genes were found to be located in a 3.5-kb DNA segment and to be organized as a typical bacterial operon. This operon could not be expressed in *E. coli* and therefore, the development of recombinant vaccines using *E. coli* expression systems was not possible (11).

The individual subunits of PT were successfully expressed in *E. coli* but these recombi-

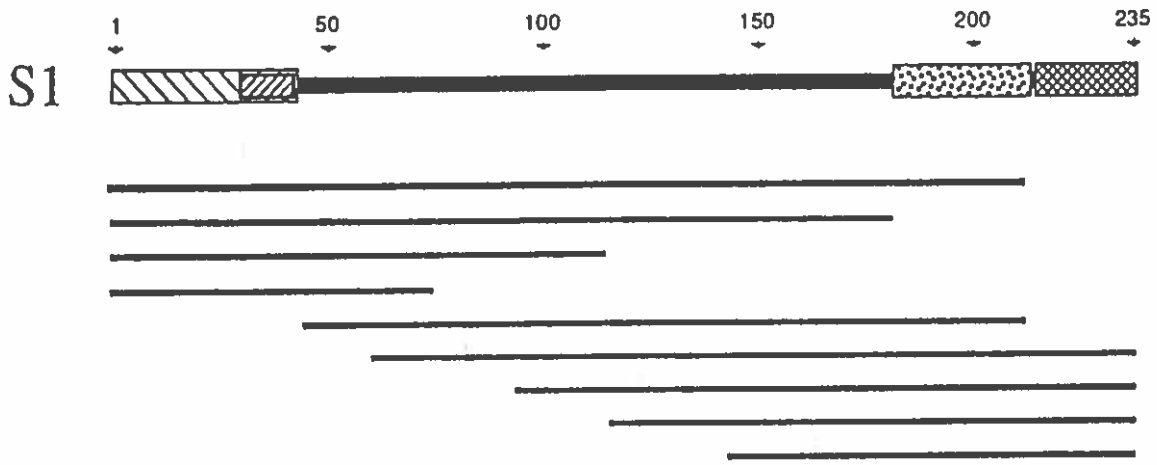


FIGURE 2. Schematic representation of the S1 subunit and the recombinant truncated fragments used in T cell proliferation assay. The three regions (1-42, 181-211 and 212-235) of S1 recognized by T cell clones are highlighted.

nant molecules were not able to induce protective antibodies, since protection was found to be mediated by conformational epitopes present in the entire assembled PT (12).

This evidence suggested that the protective vaccine should contain a PT molecule having the same B and T cell epitopes as the wild type, but made non-toxic by manipulating gene coding for the S1 subunit. To have a molecule suitable for vaccine use, we decided to modify the PT genes in the genome of *B. pertussis*, in order to obtain a molecule fully assembled, but devoid of toxicity. To this end, we needed to map the B and T cell epitopes and to identify the amino acids that were important for enzymatic activity of the S1 subunit.

Results from different laboratories had demonstrated that monoclonal antibodies capable of neutralizing the toxic activity of PT, both *in vitro* and *in vivo*, recognized the subunit S1 (13). Therefore, we collected several protective anti-S1 antibodies with the aim of mapping their epitopes. In order to investigate this issue, we constructed a number of plasmids expressing a series of aminoterminal or carboxyterminal deletions of S1 subunit and then we tested the reactivity of these molecules with neutralizing monoclonal antibodies (Figure 1). Interestingly, all protective antibodies were able to recognize the deletion mutant NCO and NRU, but did not recognize the deletion mutant BAL, indicating that the region comprised between the Nru I and Bal I sites is essential for the antibodies recognition. To our surprise, none of mutants at the 5' region, although containing the region between Bal I and Nru I, were recognized by the protective monoclonal antibodies.

Since the deletion mutant 16 A lacks only 10 aminoacids at the aminoterminus, we concluded that two discontiguous regions, containing aminoacids 1-10 (region A in Figure 1) and 124-179 (region C in Figure 2), are required for binding to the protective monoclonal antibodies.

Further experiments using all the possible combinations of the aminoterminal and carboxyterminal deletion mutants indicated that BAL could be complemented by all the aminoterminal deletion mutants, while SPH could be complemented only by 16A and 34A, suggesting that the continuity of the aminoacid sequence spanning the SPH1 site (region B in Figure 1) is also required for antibody binding. Taken together, these results indicated that

TABLE I. ***In vivo*** **and** ***in vitro*** **properties of mutant PT-9K/129G compared with wild type PT.**

Property		PT-9K/129G	PT wild type
Toxic properties of native PT			
ADP-ribosylation	(ug)	> 20	0.001
CHO-cell clustered growth	(ng/ml)	> 5000	0.005
Leucocytosis stimulation	(ug/mouse)	> 50	0.02
Anaphylaxis potentiation	(ug/mouse)	> 7.5	0.04
Enhanced insulin secretion	(ug/mouse)	> 25	< 1
Lethal dose (LD50)	(ug/kg)	> 1500	15
Non-toxic properties of native PT			
Hemagglutination	(ug/well)	0.1	0.1
T-cell mitogenicity	(ug/well)	0.1-0.3	0.1-0.3
Affinity constant (anti-S1)	Ka L/mol	6.1×10^8	2.4×10^8
Affinity constant (anti-PT)	Ka L/mol	9.8×10^9	2×10^{10}

all monoclonal antibodies recognized the same protective epitope comprising three regions that are non-contiguous in the S1 structure, but juxtaposed in the three-dimensional structure of the molecule. Therefore, these findings provided evidence that the protective epitope was conformational and that antibodies against such epitope could not be elicited by immunizing the animals with a recombinant S1 subunit that is not correctly folded within the holotoxin. (12).

To map the T cell epitopes, about 20 clones specific for PT were obtained from the peripheral blood of donors who suffered from pertussis in their childhood (14).

These T cell clones were tested for specificity with the five recombinant PT subunits. Interestingly, we found that the majority of the clones recognized S1, the subunit that in animal models has been shown to be highly immunogenic, suggesting that this subunit was immunodominant both for B and T cells.

The T cell epitopes were then mapped within the S1 subunit by using recombinant fragments representing NH2-terminal and COOH-terminal deletions of S1 (Figure 2).

This approach led to the identification of three regions of the protein as the sequences containing T cell antigenic sites: 1-42, 181-211, and 212-235.

Finally, to map the amino acids important for enzymatic activity we first tested the recombinant fragments containing amino- and carboxy-terminal deletions. The deleted protein containing the amino acids 2-180 was found to be the minimal polypeptide retaining complete enzymatic activity; thus, this region was mutagenized *in vitro*, following sequence homologies with other bacterial proteins. The substitution of the amino acids Arg 9, Asp 11, Arg 13, Trp 26, His 34 and Glu 129 was found to decrease the enzymatic activity to undetectable levels (15-17).

Therefore, each mutated S1 gene was introduced into the genome of *B. pertussis* using

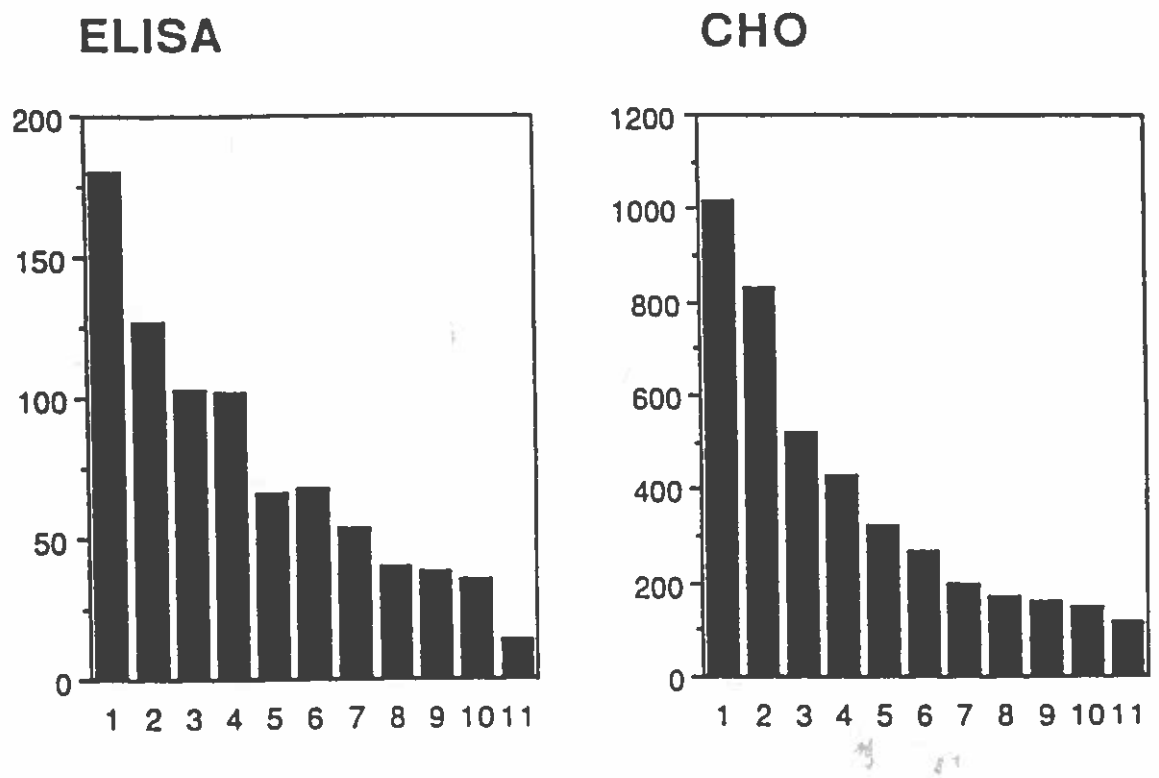


FIGURE 3. ELISA and PT neutralization titers in the CHO test of eleven acellular pertussis vaccines evaluated at NIAID unit. Vaccine number 1 is the acellular vaccine containing 15 ug of PT mutant 9K/129G per dose, whereas vaccines 2-11, provided by other manufacturers, contained a chemically detoxified PT (usually 25 ug or even more). The results shown in this figure were reported by K. Edwards *et al.*. (Poster presented at the Society for Pediatric Research Meeting, Baltimore, MD, May 6, 1992.)

homologous recombination between sequences cloned in *E. coli* and those present in a *B. pertussis* strain, in which the wild-type PT gene had been deleted.

The recombinant strains of *B. pertussis* could express and release into the culture medium the mutated forms of PT. When tested for their capacity to induce clustering effect on Chinese Hamster Ovary (CHO) cells, all mutants showed a decreased, but significant, residual toxicity ranging from 0.1% to 25% of the wild type PTx. However, the toxicity of all mutants selected was still too high to be suitable for vaccine production.

We therefore introduced two amino acid substitutions in the S1 subunit in order to further reduce the toxicity. In particular, Glu 129 was replaced by Gly and Arg 9 with Lys. The resulting double mutant, named PT-9K/129G, did not show any of the toxic effects characteristic of the native PT, such as lymphocytosis, histamine sensitivity, potentiation of anaphylaxis, whereas it maintained intact the B- and T-cell epitopes of wild type PT (Table I). This mutant was in fact capable of competing with 125 I-labeled PT as efficiently as the wild type PT for binding to a protective monoclonal antibody against the S1 subunit. In addition, PT-9K/129G was also recognized by human T-cell clones with specificity for the S1 subunit, confirming that its immunological properties had not been altered following amino acid substitution (18). When tested for its ability to induce PT-neutralizing antibodies, two injections of PT-9K/129G (0.3 ug per dose) were found to induce a neutralizing titer

of more than 1/1280, a value markedly higher than that generally observed with chemically detoxified PT.

Finally, mice immunized with the same double mutant were completely protected from the intracerebral challenge with virulent *B. pertussis* in a dose dependent manner (19). Based on all the evidence obtained in animals models, we then tested the safety and immunogenicity of PT-9K/129G mutant in adult volunteers (20, 21). Two vaccines were used: one containing only 15 ug of the mutant toxin PT-9K/129G and the other containing 5 ug of the same mutant, together with 2.5 ug of filamentous hemagglutinin (FHA) and 2.5 ug of pertactin (or 69 KD).

The National Institute for Allergy and Infectious Disease (NIAID) performed a clinical trial in the USA to compare ten acellular pertussis vaccine containing chemically detoxified PT, two acellular vaccines containing genetically detoxified PT and two whole cell vaccines. Three doses of each vaccine were given to 120 infants. The data collected indicated that the local and systemic reactions induced by the recombinant vaccines were markedly reduced when compared to those induced by the whole cell vaccine, whereas no significant differences were observed between the reactions induced by the vaccines containing the mutant and those containing chemically detoxified PT. However, both the ELISA and the CHO neutralizing titers induced by 15 ug of PT-9K/129G were much higher than those obtained by higher doses of PT that had been inactivated by a variety of chemical methods (Figure 3).

In conclusion, the data obtained in the laboratory, in animal models and in several clinical trials have shown in a definitive way that genetic detoxification of PT has produced a molecule superior to those obtained by chemical treatment in that it is completely devoid of toxicity, but still retains all the immunological properties of the native toxin.

References

1. Muller, A.S., Leeuvenburgh, and Pratt, D.S. (1986) *Bull. WHO 64:*321.
2. Brennan, M.J., Burns, D.L., Meade, B.D., Shahin, R.D. and Manclark, C.R. (1992) In: *Vaccines*, New Approaches to Immunological Problems, (R.W. Ellis, ed), pp. 23–52, Butterworth-Heinemann, Boston, London.
3. Kendrick, P. and Eldering, G. (1936) *Am. J. Public Health 26:*8.
4. Ross, E.M., Alderskade, R., Bellman, M.H. and Rawson, N.S.B. (1981) *Br. Med. J. 282:*1595.
5. Robinson, A., Irons, L.I. and Ashworth, L.A.E. (1985) *Vaccine 3:*11.
6. Ad Hoc Group for the Study of Pertussis Vaccine. (1988) *Lancet i:*955.
7. Storsaeter, J., Olin, P., Renemar, B., Lagergärd, T., Norberg, R., Romanus, V. and Tiru, M. (1988) *Pediatr. Infect. Dis. 7:*637.
8. Tamura, M., Nagimori, K., Murai, S., Yajima, M., Ito, K., Katada, T., Ui, M. and Ishii, S. (1982) *Biochemistry 21:*5516–5522.
9. Nicosia, A., Perugini, M., Francini, C., Casagli, M.C., Borri, M.G., Antoni, G., Almoni, M., Neri, P., Ratti, G. and Rappuoli, R. (1986) *Proc. Natl. Acad. Sci. USA 83:*4631–4635.
10. Locth, C. and Keith, J. (1986) *Science 232:*1258–1264.
11. Nicosia, A. and Rappuoli, R. (1987) *Infect. Imm. 55:*963–967.
12. Bartoloni, A., Pizza, M.G., Bigio, M., Nucci, D., Ashwort, L.A., Irons, L.I., Robinson, A., Burns, D., Manclark, C.R., Sato, H. and Rappuoli, R. (1988) *Biotechnology 6:*704–712.
13. Sato, H., Ito, A., Chiba, J. and Sato, Y. (1984) *Infect. Immun. 46:*422–428.
14. De Magistris, M.T., Romano, M., Bartoloni, A. and Tagliabue, A. (1989) *J. Exp. Med. 169:*1519–1532.

15. Burnette, J.T. (1988) *Science 242:*72-74.
16. Barbieri, J.T. and Cortina, G. (1988) *Infect. Immun. 56:*1934-1941.
17. Pizza, M.G., Bartoloni, A., Prugnola, A., Silvestri, S. and Rappuoli, R. (1988) *Proc. Natl. Acad. Sci. USA 85:*7521-7525.
18. Pizza, M.G., Covacci, A., Bartoloni, A., Perugini, M., Nencioni, L., De Magistris, M.T., Villa, L., Nucci, D., Manetti, R., Bugnoli, M., Giovannoni, F., Olivieri, R., Barbieri, J.T., Sato, H. and Rappuoli, R. (1989) *Science 246:*497-500.
19. Nencioni, L., Pizza, M.G., Bugnoli, M., De Magistris, M.T., Di Tommaso, A., Giovannoni, F., Matteucci, G., Nucci, D., Olivieri, R., Pileri, P., Presentini, R., Villa, L., Kreeftenberg, J.G., Silvestri, S., Tagliabue, A. and Rappuoli, R. (1990) *Infect. Immun. 58:*1308-1315.
20. Podda, A., Nencioni, L., De Magistris, M.T., Di Tommaso, A., Bossù, P., Nuti, S., Pileri, P., Peppoloni, S., Bugnoli, M., Ruggiero, P., Marsili, I., D'Errico, A., Tagliabue, A. and Rappuoli, R. (1990) *J. Exp. Med. 172:*861-868.
21. Podda, A., Nencioni, L., Marsili, I., Peppoloni, S., Volpini, G., Donati, D., Di Tommaso, A., De Magistris, M.T. and Rappuoli, R. (1991) *Vaccine 9:*741-745.

Physiol. Phys. Chem. & Med. NMR (1995) 27:363-368

Phosphorylation of Acidic Peptides by Protein Kinase CKII

Elena Cardellini, Antonella Angiolillo and Gian Luigi Gianfranceschi

Dipartimento di Biologia Cellulare e Molecolare Sez. di Fisiologia e Biofisica, Università degli Studi di Perugia, Perugia, Italy

SYNTHETIC PEPTIDES have been designed on the basis of native peptides characteristics as described in the previous paper (1). Because native peptides are phosphorylated at Ser residue, the amino acid sequence of the synthetic peptides was examined to identify potential consensus sites for known kinases: peptide 5 (Table I) was found to contain the protein kinase CKII (pCKII) consensus site Glu-Asp-Ser-Asp-Glu-Glu. Protein kinase CKII is an ubiquitous enzyme able to phosphorylate Ser/Thr residues followed by a cluster of acidic amino acids: a necessary and sufficient condition for phosphorylation is the presence of a Glu or Asp residue at position +3 with respect to the target amino acid (2).

Peptide 5 is shown to be a good substrate for pCKII, isolated and purified from calf thymus (3), as indicated by the km value of 2×10^{-4} M (Table I).

The other four peptides, which do not contain the consensus site for pCKII, could also be phosphorylated when incubated with the purified enzyme (4,5), even though the affinity was low when compared with that of peptide 5, as shown by higher km values and lower phosphorylation rates (Table I). The specificity of phosphorylation efficiency was demonstrated by utilizing some derivatives of peptide 1: the data reported in Table II show that three factors act as positive determinants: (i) the Glu or Asp residue on the N-terminal position of Ser residue; (ii) the Asn residue on the C-terminal position of Ser residue; (iii) absence of positive charge at the N-terminal, eg. presence of pyroGlu.

The possibility that sequences with the characteristics described above could represent phosphorylation sites *in vivo* is supported by the presence of the sequence of peptide 1 (where pyroGlu was replaced by Gln) or of very similar sequences in several proteins: the most interesting examples are shown in Table III.

Phosphorylation of peptides 2 and 3 (Table I) also follows the rules observed for peptide 1. The phosphorylation site of peptide 3 is contained in the human interleukin 1 β precursor.

TABLE I. Phosphorylation of synthetic peptides by calf thymus protein kinase CKII.

	Substrate	Phosphorylation rate	k_m (M)
1.	pyroGlu-Ala-Glu-Ser-Asn	100	4×10^{-3}
2.	pyroGlu-Ala-Gly-Glu-Ser-Glu-Asp	18	10^{-2}
3.	pyroGlu-Ala-Gly-Glu-Glu-Ser-Asn	94	2×10^{-3}
4.	pyroGlu-Ala-Gly-Glu-Glu-Glu-Glu-Ser-Asn	37	5×10^{-3}
5.	pyroGlu-Ala-Gly-Glu-Asp-Ser-Asp-Glu-Glu-Asn	620	2×10^{-4}

Phosphorylation rates are relative percent values taking phosphorylation rate of peptide 1 as 100%

TABLE II. Phosphorylation of peptide 1 derivatives by calf thymus protein kinase CKII.

	Substrate	Phosphorylation rate
1.	pyroGlu-Ala-Glu-Ser-Asn	100
6.	pyroGlu-Ala-Gln-Ser-Asn	55
7.	Glu-Ala-Glu-Ser-Asn	66
8.	pyroGlu-Ala-Glu-Ser-Asn-Ala	35
9.	pyroGlu-Ala-Glu-Ser-Leu	18
10.	ac-Ser-NH_2	0
11.	pyroGlu-Ala-Glu-Ser	0
12.	pyroGlu-Glu-Asp-Ser-Gly	130
13.	pyroGlu-Glu-Gly-Ser-Asp	65

The peptide Val^{163}-Gln-Gly-Glu-Glu-Ser-Asn-Asp-Lys^{171} contained in the interleukin 1β precursor could be phosphorylated by pCKII and some of its derivatives were synthesized in order to identify the conditions able to facilitate the phosphorylation. As shown in Table IV, the presence of an Asn residue at the C-terminal of Ser acts as strong positive determinant.

Phosphorylation of peptides 4 and 5 (Table I) fits the rules usually described in the literature: a cluster of acidic amino acids downstream of Ser strongly increases the affinity of pCKII: e.g. see the affinity of peptide 5 with respect to that of peptide 4.

The phosphorylation site of peptide 5 or of similar sequences was found in many proteins,

TABLE III. Proteins containing the pCKII consensus sequence Gln-Ala-Glu-Ser-Asn or very similar sequence.

Sequence	Protein	aa residue
Asn-Ala-Glu-Ser-Asn	virA protein-Agrobacterium tumefaciens plasmid pTiAg162	512
Ser-Ala-Glu-Ser-Asn	phycocianin hypothetical linker polypeptide	253
Glu-Ala-Glu-Ser-Asn	tubulin beta-2 chain, fruit fly	410
Glu-Ala-Glu-Ser-Asn	tubulin beta-3 chain, fruit fly	416
Gln-Glu-Glu-Ser-Asn	human coagulation factor IX precursor	96
Gln-Ala-Glu-Ser-Asp	Na/K transporting ATPase alfa chain precursor, rat	826
Gln-Gly-Glu-Ser-Asn	human somatoliberin precursor	62
Glu-Ala-Glu-Ser-Asn	tubulin beta chain, human	410
Glu-Ala-Glu-Ser-Asn	tubulin beta-2 chain fragment, mouse	286
Glu-Ala-Glu-Ser-Asn	tubulin beta-3 chain, mouse	410
Glu-Ala-Glu-Ser-Asn	tubulin beta-4 chain, mouse	410
Glu-Ala-Glu-Ser-Asn	tubulin beta-5 chain, mouse	410
Asn-Ala-Glu-Ser-Asn	bovine fetuin (fragments)	61
Gln-Ala-Glu-Ser-Glu	myosin heavy chain, skeletal muscle, rat	893
Ala-Ala-Glu-Ser-Asn	cell division control protein 37, yeast	149
Gln-Ser-Glu-Ser-Asn	C-reactive protein, plaice	9
Gln-Ala-Asn-Ser-Asn	DNA directed RNA polymerase-beta chain (version 1), E. coli	618
Gln-Glu-Glu-Ser-Asn	bovine coagulation factor IX	50
Leu-Ala-Glu-Ser-Asn	human (2′-5′) oligo (A) synthetase, isoenzyme E18	346
Gln-Pro-Glu-Ser-Asn	transforming protein (alfa), mouse	52
Asp-Ala-Glu-Ser-Asn	protein A, E. coli	144
Met-Ala-Glu-Ser-Asn	capsid assembly protein, bacteriophage T7	1

TABLE IV. Phosphorylation of the interleukin 1β peptide and some of its derivatives.

	Substrate	phosphorylation rate
1.	pyroGlu-Ala-Glu-Ser-Asn	100
14.	Val-Gln-Gly-Glu-Glu-Ser-Asn-Asp-Lys	26
15.	Gly-Glu-Glu-Ser-Asn-Asp	26
16.	Glu-Glu-Ser-Asn-Asp	34
17.	Gly-Glu-Glu-Ser-Asn	292

TABLE V. Proteins containing the pCKII consensus sequence Asp/Glu-Ser/Thr-Asp/Glu-Asp/Glu-Asp/Glu or very similar sequence.

Sequence	Proteins	aa residue
Ala-Ile-Ser-Pro-Asp-Asp-Ser-Asp-Glu-Glu-Asn	RNA polymerase II sub.215 kda	1921
Ile-Ser-Ser-Ser-Glu-Asp-Ser-Asp-Ala-Glu-Asn	PML	557
Glu-Ala-Gly-Phe-Pro-Pro-Ser-Asp-Asp-Glu-Asp-Glu	Ad Ela	126
Glu-Glu-Met-Pro-Ser-Ser-Asp-Asp-Glu-Ala-Thr	SV40 LT	107
Glu-Gln-Leu-Ser-Asp-Ser-Glu-Glu-Glu-Asn-Asp	HPV 18E7	29
Asn-Asp-Ser-Ser-Glu-Glu-Glu-Asp-Glu	HPV 16E7	29
Val-Asp-Ser-Ser-Glu-Asp-Glu-Val-Asp	HPV 6bE7	30
Val-Gly-Pro-Asp-Ser-Asp	p53	384
Thr-Ser-Ser-Asp-Ser-Glu-Glu-Glu-Gln-Glu	human Myc	248
Ser-Ser-Asp-Thr-Glu-Glu-Asn-Val	human Myc	347
Thr-Ser-Ser-Asp-Ser-Glu-Glu-Glu-Gln-Glu	avian Myc	224
Thr-Ser-Asp-Ser-Glu-Glu-Asn-Asp	avian Myc	323
Thr-Pro-Glu-Ser-Glu-Glu-Ala	c-Fos	230
Ser- Ile-Tyr-Ser-Ser-Asp-Glu-Asp-Asp-Glu	human myb	7

TABLE VI. Phosphorylation of nonhistone proteins, contained in calf thymus chromatin extracts, by purified pCKII in presence of peptide 18.

peptide 18 μg	Protein phosphorylation
0	100
2	41
10	36
30	27

several of which represent transcription factors (Table V). The octapeptide pyroGlu-Asp-Asp-Ser-Asp-Glu-Glu-Asn (peptide 18) containing the heptapeptide of the RNA polymerase II largest subunit (Table V) was synthesized and used as substrate for pCKII: its phosphorylation rate was 709 taking the phosphorylation rate of peptide 1 as 100. Because of its high affinity for pCKII, peptide 18 could potentially represent a competitor in a protein phosphorylation assay: phosphorylation by pCKII of nonhistone proteins, contained in calf thymus extracts, was decreased after addition of the peptide (Table VI).

TABLE VII. Calf thymus DNA transcription by calf thymus RNA polymerase II in the presence of phosphorylated peptide 18.

peptide 18 (ng)	transcription rate
0	100
4	100
8	100
12	100
25	41
50	12
100	6
200	3

As described in the previous paper (1), native peptides are able to inhibit DNA transcription, therefore peptide 18 was utilized to check if this inhibition could be due to a binding to DNA. Phosphorylated peptide 18 binds calf thymus DNA only in the presence of Mg^{2+}: 3 nmoles of calf thymus DNA base pairs were able to bind 1 pmole of peptide; the unphosphorylated peptide could not bind to DNA. The DNA-peptide complex was used as template in a DNA transcription by RNA polymerase II purified from calf thymus: the results showed that the phosphopeptide can strongly inhibit transcription in a dose dependent manner (Table VII) and this effect does not change when transcription factors are added, indicating a possible phosphopeptide inhibiting role directly on the DNA-RNA polymerase complex (6).

In this paper data on phosphorylation of acidic synthetic peptides were reviewed and summarized: they indicate a potential role of acidic phosphorylated peptides in regulation of *in vivo* DNA transcription. The finding that these peptides represent domains of proteins involved in DNA transcription supports the hypothesis that acidic phosphorylated peptides possibly derive from protein processing and can regulate such proteins synthesis and function.

The authors wish to thank Dr. Felici Franco (IRBM; Pomezia, Italy) for computerized research of protein sequences by utilizing the GCG Sequence Analysis Software Package (Genetics Computer Group, 1991, program manual for the GCG package, version 7, April 1991, 575 Science Drive, Madison, WI 53711).

References

1. Lugaro, G., De Angelis, L., Giavarini, F., Marsili, V., Mancinelli, L., Castigli, E. and Gianfranceschi, G.L. (1995) *Physiol. Chem. Phys. & Med. NMR 27:*345–349.
2. Pinna, L.A. (1990) *Biochim. Biophys. Acta 1054:*267–284.
3. Gianfranceschi, G.L., Cardellini, E., Piccinini, G., Bramucci, M., Miano, A., Maccari, E., Amici, D. and Durban, E. (1989) *Physiol. Chem. Phys. & Med. NMR 21:*289–294.
4. Chillemi, F., Lugaro, G., Boari, D., Cardellini, E., Bramucci, M., Miano, A., Amici, D., Gianfranceschi, G.L. and Durban, E. (1991) *FEBS Lett. 291:*67–70.
5. Angiolillo, A., Bramucci, M., Marsili, V., Panara, F., Miano, A., Amici, D. and Gianfranceschi, G.L. (1993) *Mol. Cell. Biochem. 125:*65–72.
6. Angiolillo, A., Desgro, A., Marsili, V., Panara, F. and Gianfranceschi, G.L. (1993) *Experientia 49:*902–905.

Physiol. Chem. Phys. & Med. NMR (1995) 27:369-376

Influence of Cell Differentiation and Protein Kinase C Activation on Sub-Cellular Distribution of β-N-Acetylhexosaminidases of HL 60 Cells

Carla Emiliani, Sabata Martino, John L. Stirling[1] and Aldo Orlacchio

Dipartimento di Biologia Cellulare e Molecolare, Università degli Studi di Perugia, via del Giochetto, 06126 Perugia, Italy and [1]Division of Life Sciences, King's College London, Kensington Campus, Campden Hill, London, W87AH, U.K.

Abstract: There have been several accounts regarding the alterations of the lysosomal enzyme β-N-acetylhexosaminidase in human leukaemic cells. In addition to Hex A (αβ) and Hex B (ββ) forms, leukaemic cells contain a third isoenzyme displaying many characteristics in common with Hex S, the αα dimer representing the residual activity in patients with Sandhoff's disease. In the human leukaemic cell line HL 60, A (αβ) and S (αα) are the most abundant forms. Sub-cellular fractionation of HL 60 cells showed that both A and S forms were present in the lysosomal and post-lysosomal fractions, however, a proportion of activity was found to be associated with the plasma membrane.

The phorbol ester 12-O-tetra-decanoylphorbol-13-acetate (TPA) exerts complex effects on the physiology of HL 60 cells, leading to cell differentiation along the macrophage pathway and including activation of Protein Kinase C (PKC). In order to assess the extent to which cell differentiation and PKC activation plays a role in modulating the expression of hexosaminidase during cell differentiation, we treated HL 60 cells with TPA and in parallel with the more specific activator of PKC, 1-oleoyl-2-acetyl diglycerol (OAG) which does not cause cell differentiation. We observed that 24 h exposure of HL 60 cells to TPA or OAG produced significant modification of the hexosaminidase isoenzyme pattern of HL 60 cells. The most remarkable effect was seen in both cases in the plasma membrane fraction. Taken together, our results suggest a correlation between hexosaminidase expression and kinase(s) activation.

THE LYSOSOMAL GLYCOHYDROLASE β-N-acetylhexosaminidase (E.C.3.2.1.52, Hex) is present as two isoenzymes A and B in normal human cells and tissues. Two subunits, α and β, are required for the formation of Hex A (αβ), while B is a dimer of β-subunits

(Mahuran *et al.*, 1985). Hex A accounts for about 60–70% of the total Hex activity and Hex B for 30–40%. cDNAs for both genes have been cloned and sequenced, the genes mapped to chromosomes 15q (HEX A) and 5q (HEX B) and the exon/intron structures of both described (Proia and Soravia, 1987; Proia, 1988). Both subunits are synthesized as precursors that undergo a variety of post-translational modifications that include removal of the signal peptides, N-glycosilation, dimer formation to form A (αβ) or B (αα) which seems to be a pre-requisite for their transport to lysosomes (Proia *et al.*, 1984; von Figura and Hasilik, 1986; Little *et al.*, 1988). In normal cells the αα dimer is either unstable or not formed at appreciable rates, or otherwise unable to reach the lysosomes for reasons that are not entirely clear (Proia *et al.*, 1984). Formation of the αα dimer does take place to some extent in Sandhoff's disease, a variant of GM2-gangliosidosis that results from mutations of the Hex B gene (Proia *et al.*, 1984) and is also observed when α-subunits are over expressed in COS cells transfected with α-subunit cDNA (Gluzman, 1981; Sonderfeld-Fresko and Proia, 1989).

Recently attention has been paid to the variations of Hex isoenzymes in leukaemic cells. An αα-dimer of Hex, with biochemical and immunological properties in common with Hex S, Sandhoff's disease isoenzyme, has been described in a variety of fresh and cultured leukaemic cells (see for review Emiliani and Orlacchio, 1992). In the promyelocytic cell line HL 60 (Collins, 1987), while the B (ββ) form is reduced to 5% of the total activity, there is, in addition to Hex A (αβ), a relatively large amount of Hex S (αα), which represents 40% of Hex activity measured with the substrate 4-methylumbelliferyl-β-N-acetylglucosamine (MUG), hydrolyzed by both the α- and β-subunits of the enzyme, and about 70% of the Hex activity measured with 4-methylumbelliferyl-β-N-acetylglucosamine-6-sulphate (MUGS) the substrate specific for active α-subunit (Emiliani *et al.*, 1990a). This form displayed an increased stability compared to Hex S from Sandhoff's disease and its abundance is modulated by cell differentiation. In fact differentiation of HL 60 cells along the pathway towards granulocytes, induced by treating cells in culture with dimethyl sulphoxide (DMSO), produced a modified Hex isoenzyme profile in which A became the major form, whereas the levels of Hex S were greatly decreased and those of Hex B increased (Emiliani *et al.*, 1990b). The resulting Hex profile closely resembles that seen in normal granulocytes.

In order to clarify the mechanisms leading to the anomalous expression of Hex in HL 60 cells, we have analyzed the sub-cellular distribution of Hex in HL 60 cells. We found that combination of anionic exchange DEAE-cellulose chromatography and assays of Hex activity with the two fluorogenic substrates MUG and MUGS provided a useful approach for revealing the distribution and subunit structures of Hex isoenzymes, including the minor forms appearing in HL 60 sub-cellular fractions.

Cell fractionation was performed by differential centrifugation as described by Martino *et al.*, (1995). Briefly, cells were harvested and resuspended in 0.25 M-sucrose, then homogenized in a Potter Elveheim type homogenizer until more than 90% of the cells were disrupted. Differential centrifugation of the homogenate was performed at 800g for 10 min at 4ºC in the AJ20 rotor of a Beckman J2-21 centrifuge to sediment the nuclear fraction. The supernatant was then centrifuged at 13.000g for 15 min to sediment the lysosomal fraction and the supernatant (post-lysosomal fraction) from this step was decanted. Fractions enriched in plasma membranes were obtained by resuspending the 800g pellet in the upper phase of the two-phase polymer system of Brunette and Till (1971). Membranes which banded at the interface were recovered and washed in 10 mM phosphate buffer, pH 6.0

TABLE I. Distribution of β-N-acetylhexosaminidase activity in sub-cellular fractions of HL 60 cells.

Sub-cellular fractions*	RSA values**			
	β-Hexosaminidase		β-Galactosidase	Alkaline phosphatase
	(MUG)	(MUGS)		
Lysosomal	2.45	2.63	3.60	2.15
Post-Lysosomal	0.78	0.78	0.64	0.19
Plasma Membrane	1.05	1.06	0.23	10.00

Values are the means of 4 independent experiments.
* Cell fractionation was performed by differential centrifugation as described in text.
** RSA is the relative specific activity (De Duve *et al.*, 1955). It is the percentage of total activity in a given fraction divided by the percentage of total proteins in the fraction.

containing the detergent Nonidet P-40. The distribution of Hex activity in comparison with the activity of the alkaline phosphatase, a marker for plasma membrane, and β-galactosidase, used as a marker of the lysosomal fraction, are reported in Table I. As seen in Table I, the Hex activity is most abundant in the lysosomal fraction, but part of this activity was also detected in the plasma membrane fraction. On the contrary, almost all β-galactosidase activity was confined to the lysosomal fraction. DEAE cellulose chromatography was used to separate the Hex isoenzymes from the lysosomal, post-lysosomal and membrane fractions. The proportions of B, A and S forms are reported in Figure 1. Two aspects of these results should be emphasized. Firstly the presence of Hex S, in addition to Hex A, in the lysosomal fraction gives the first evidence of transport of Hex S (αα dimer) to lysosomes. Evidence from Sandhoff disease fibroblasts suggests that transport of the αα dimer to lysosomes does not take place in this cell type (Proia *et al.*, 1984). Secondly a proportion of Hex activity is associated with the plasma membrane fraction. A detergent (Nonidet P-40) was required to solubilize the activity.

Having found that the sub-cellular localization of Hex isoenzymes differs from that of other lysosomal enzymes such as β-galactosidase, we wondered if cell differentiation may influence the expression and localization of Hex in HL 60 cells. For this reason we treated HL 60 cells with the phorbol ester TPA which causes them to differentiate along the macrophage pathway. TPA exerts complex effects on the physiology of HL 60 cells, leading to cell differentiation, including activation of PKC. Phorbol esters including TPA are known to be potent activators of PKC (Castagna *et al.*, 1982; Feuerstein and Cooper, 1983; Niedel *et al.*, 1983; Feuerstein and Cooper, 1984). The fundamental role of PKC on HL 60 cell differentiation is demonstrated by the fact that defective translocation of PKC in multidrug resistant HL 60 cells confers loss of ability of the cells to differentiate as consequence of TPA-treatment (Slapak *et al.*, 1993). In order to assess the extent to which PKC activation plays a role in modulating the expression of Hex duing cell differentiation, we treated HL 60 cells, in parallel with the more specific activator of PKC, OAG, which does not cause cell differentiation (Kreutter *et al.*, 1985). HL 60 cells were cultured in RPMI 1640 medium containing 10% (v/v) heat-inactivated fetal calf serum. When the cell concentration was

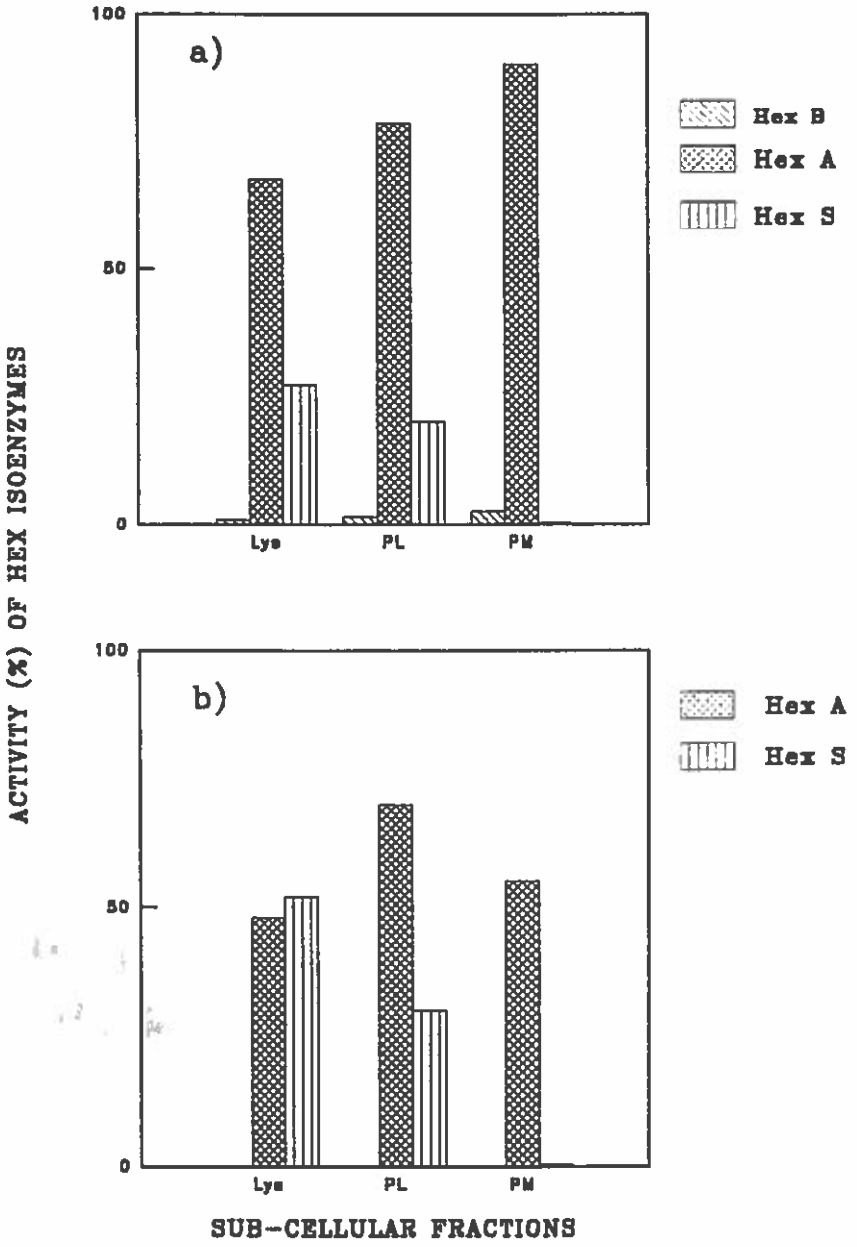


FIGURE 1. Proportions of β-N-acetylhexosaminidases B, A and S in the sub-cellular fractions of HL 60 cells.

Cell fractionation was performed by differential centrifugation as described by Martino *et al.* (1995). Briefly, cells were harvested and resuspended in 0.25 M-sucrose, then homogenized. Differential centrifugation of the homogenate was performed at 800g to sediment the nuclear fraction; the supernatant was then centrifuged at 13.000g for 15 min to sediment the lysosomal fraction (Lys) and the supernatant (post-lysosomal -PL-fraction) from this step was decanted. Fractions enriched in plasma membranes (PM) were obtained by resuspending the 800g pellet in the upper phase of the two-phase polymer system of Brunette and Till (1971). Each fraction was applied to 1 ml DEAE-cellulose column, which was developed as described in Figure 2. Enzymatically active peaks, corresponding to Hex B, A and S in order of their elution from the column, were pooled. The activity of each isoenzyme is expressed as percentage of the total activity recovered from the column. Enzyme activity was measured by using the substrates MUG (a) and MUGS (b).

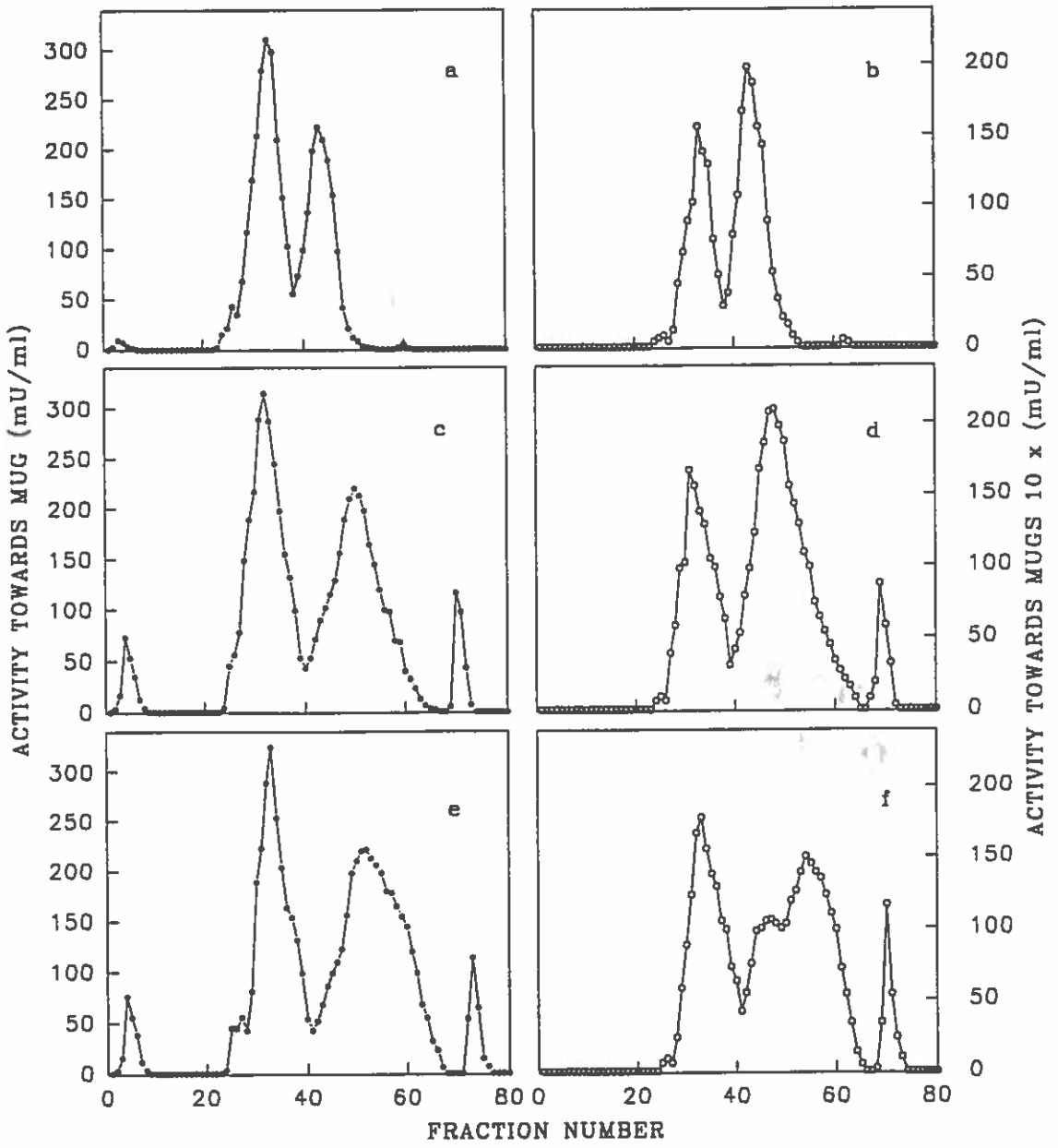


FIGURE 2. β-N-acetylhexosaminidases of HL 60 cells grown for 24 h in the absence and in the presence of TPA and OAG.

Extracts from 10^7 untreated (a, b), TPA treated (c, d) or OAG treated (e, f) HL 60 cells were applied to a 1 ml DEAE-cellulose column equilibrated wtih 10 mM-sodium phosphate buffer, pH 6.0. Enzyme activity retained by the column was eluted in a linear gradient of NaCl that reached a concentration of 0.5 M in 30 ml of buffer. Fractions (0.5 ml) were assayed for activity towards the substrate MUG (•) or MUGS (O).

1×10^6/ml, TPA or OAG were added to give a final concentration of 20 nM (TPA) and 20 μg/ml (OAG). Treated and control cells were harvested after 24 h of incubation. Cell growth was determined as well as their phagocytic ability. After 24 h of TPA treatment, a high proportion of cells (50%) became sticky and adhered to the surface of tissue culture flasks.

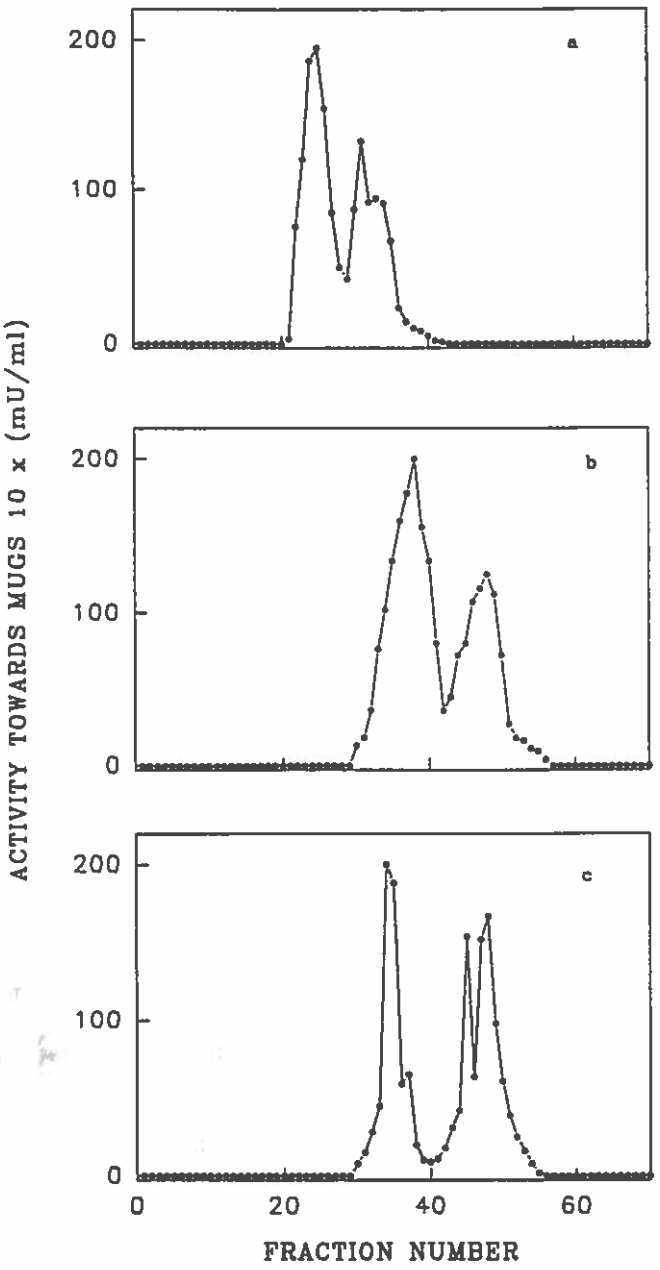


FIGURE 3. Distribution of β-N-acetylhexosaminidases isoenzymes in plasma membranes of HL 60 cells grown for 24 h in the absence and presence of TPA or OAG.

Cell membranes from 10^7 untreated (a), TPA treated (b) or OAG treated (c) HL 60 cells were prepared as described. Each fraction was applied to 1 ml DEAE-cellulose column, which was developed as described in Figure 2. Fractions (0.5 ml) were assayed for activity towards the substrate MUGS.

In contrast, there were no obvious changes in the behavior of HL 60 cells treated with OAG and all cells remained in suspension without evidence of clumping. DEAE-cellulose chromatography, performed on extracts of controls, 24 h TPA treated or 24 h OAG treated HL 60 cells, are shown in Figure 2. The isoenzyme profiles, detected using both MUG and MUGS substrates, revealed that as in the controls, the predominant enzymes in treated cells are Hex

A and Hex S. The main difference between treated and untreated cells was in the proportion of NaCl required to elute the two major forms. In fact, in TPA treated cells Hex A was eluted at a lower concentration of NaCl than in the control and a proportion of the Hex S peak was eluted at higher salt concentration indicating that its net charge was more acidic than that of the control (Figure 2,b). The effect of OAG, which is thought to be a more specific activation of PKC, on the HL 60 Hex profile was similar to that of TPA over 24 h (Figure 2,c). This led us to hypothesize that PKC activation has a specific role in modulating the expression of Hex during cell differentiation.

To clarify this point, homogenates of the control, 24 h TPA or OAG treated HL 60 cells were made in buffered 0.25 M sucrose and separated by differential centrifugation as described into three fractions: plasma membrane, lysosomal and post-lysosomal. The hexosaminidase isoenzyme content of each fraction was characterized by chromatography on DEAE-cellulose and assaying each fraction with the two substrates MUG and MUGS, as above. The most appreciable effect of TPA treatment for 24 h was on the plasma membrane Hex isoenzymes (Figure 3). While only subtle changes were seen in the lysosomal and post lysosomal fractions of TPA treated cells, when compared with control cells, in the plasma membrane fraction there were remarkable changes of the isoenzyme profile. In particular, as shown in Figure 3b, we observed the appearance of the S form in the membrane and the entire membrane Hex isoenzyme pattern exhibited an acidic shift compared with that of untreated cells (Figure 3a). An increase of Hex B activity and the appearance of an extra-acidic peak, which required 1 M NaCl for elution from the DEAE-column was also observed. An even more exaggerated acidic shift in the A and S forms of the membrane fraction was caused by OAG treatment of HL 60 cells (Figure 3c). As for TPA, no appreciable changes in the distribution of hexosaminidase isoenzymes in either the lysosomal or soluble fractions were detected as a consequence of OAG treatment. All these data indicate that over 24 h exposure, the main effect of TPA is on the plasma membrane Hex activity. The evidence that, over this time of exposure, OAG treatment induced a comparable effect, strongly suggests an involvement of PKC in mechanisms regulating Hex activity during cell differentiation of HL 60.

We have evidence that the described effects are transient and that exposure of the cells to TPA restores the Hex isoenzyme pattern of fully differentiated cells (Martino *et al.* manuscript in preparation). Nevertheless, the membrane Hex isoenzyme pattern remains more acidic and the Hex S still present even after 72 h of exposure to TPA. All these observations indicate that the expression of Hex in HL 60 cells is modulated by cell differentiation and that PKC activation and related processes play a specific role.

TPA is known to affect gene expression through the binding of the AP-1 transcription factor to genes with promoters having TPA response elements (Lee *et al.*, 1987). Neote *et al.* (1988) showed that the Hex B gene has such elements but whether they are functional is unknown. It seems possible however that at least part of the increased abundance of Hex B in HL 60 cells treated with TPA could result from increased transcription of the Hex B gene. In support of our hypothesis, the analysis of the α- and β-subunit sequences revealed that they both contain a number of motifs that could act as substrates for PKC but as yet there is no direct evidence that they function as phosphorylation sites *in vivo*. Pulse-chase experiments with ^{32}P labeled phosphate have shown that α-subunits are more heavily phosphorylated than β-subunits in normal fibroblasts growing in culture (Hasilik and Neufeld, 1980). While most of this was thought to be in the form of mannose-6-phosphate, it is now known

(O'Dowd *et al.*, 1988) that α-subunits have a single oligosaccharide chain that is a potential substrate for UDP-glcNAc phosphotransferase and β-subunits have two such oligosaccharides. There is therefore a distinct possibility that the difference in intensity of labeling is caused by protein phosphorylation.

More work must be done before showing definitively that Hex is a substrate protein for PKC or related kinases. However we feel that the identification of Hex activity on HL 60 plasma membranes and its susceptibility to compounds activating PKC is an observation that merits further investigation and will lead to a better understanding of the mechanisms of regulation of Hex activity during growth and differentiation of leukaemic cells.

Work supported by Italian Consiglio Nazionale delle Ricerche, P.F. A.C.R.O, SP 4, contract # 94.022.08 PF39 and P.F. Ingegneria Genetica, contract # 94.00039 PF99.

References

Brunette, D.M. and Till, J.E. (1971) *J. Membrane Biology 5:*215-224.

Castagna, M., Takay, Y., Kaibuchi, K., Sano, K., Kikkawa, U. and Nishizuka, Y. (1982) *J. Biol. Chem. 257:*7847-7851.

Collins, S.J. (1987) *Blood 70:*1233-1244.

De Duve, C., Pressman, B.C., Giannetto, R., Wattiaux, R. and Applemans, F. (1955) *Biochem. J. 60:*604-617.

Emiliani, C., Beccari, T., Tabilio, A., Orlacchio, A., Hosseini, R. and Stirling, J.L. (1990a) *Biochem. J. 267:*111-117.

Emiliani, C., Falzetti, F., Orlacchio, A. and Stirling, J.L. (1990b) *Biochem. J. 272:*211-215.

Emiliani, C. and Orlacchio, A. (1992) Minireview, *Int. J. Biochem. 24:*539-544.

Feuerstein, N. and Cooper, H.L. (1983) *J. Biol. Chem. 258:*10786-10793.

Feuerstein, N. and Cooper, H.L. (1984) *J. Biol. Chem. 259:*2782-2788.

Gluzman, Y. (1981) *Cell 23:*175-182.

Hasilik, A. and Neufeld, E.F. (1980) *J. Biol. Chem. 255:*4946-4950.

Kreutter, D., Caldwell, A.B. and Morin, J.M. (1985) *J. Biol. Chem. 260:*5979-5984.

Lee, W., Mitchell, P. and Tjian, R. (1987) *Cell 49:*741-752.

Little, L.E., Lau, M.M.H., Quon, D.V.K., Fowler, A.V. and Neufeld, E.F. (1988) *J. Biol. Chem. 263:*4288-4292.

Martino, S., Emiliani, C., Orlacchio, A., Hosseini, R. and Stirling, J.L. (1995) *Biochim. Biophys. Acta 1243:*489-495.

Mahuran, D.J., Novak, A. and Lowden, J.A. (1985) In: *Isoenzymes, Current Topics in Biological and Medical Research, vol. 12,* Alan Liss, Inc., New York, pp. 229-288.

Neote, K., Bapat, B., Dumbrille Ross, A., Troxel, C., Schuster, S.M. Mahuran, D.J. and Gravel, R.A. (1988) *Genomics 3:*279-286.

Niedel, J.E., Kuhn, L. and Vandenbark, J.R (1983) *Proc. Natl. Acad. Sci. U.S.A. 80:*36-40.

O'Dowd, B.F., Cumming, D.A., Gravel, R.A. and Mahuran, D. (1988) *Biochemistry 27:*5216-5226.

Proia, R.L., d'Azzo, A. and Neufeld, E. (1984) *J. Biol. Chem. 259:*3350-3354.

Proia, R.L. and Soravia, E. (1987) *J. Biol. Chem. 262:*5677-5681.

Proia, R.L. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85:*1883-1887.

Slapak, C.A., Kharbanda, A.S. and Kufe, D.W. (1993) *J. Biol. Chem. 268:*12267-12273.

Sonderfeld-Fresko, S. and Proia, R.L. (1989) *J. Biol. Chem. 264:*7692-7697.

von Figura, K. and Hasilik, A. (1986) *Annu. Rev. Biochim. 55:*167-193.

von Figura, K., Konzelmann, E., Neufeld, E.F., Kaback, M.M. and Suzuki, K. (1989) In: *The Metabolic Basis of Inherited Disease* (C.R. Scriver, A.L. Beaudet, W.S. Sly, and Valle, eds), McGraw-Hill Inc., New York, pp. 1807-1842.

Physiol. Chem. Phys. & Med. NMR (1995) 27:377-400

Intermediate Filaments and Gene Regulation

Peter Traub

Max-Planck-Institut für Zellbiologie, Rosenhof, 68526 Ladenburg/Heidelberg, Germany

Abstract: The biological role of intermediate filaments (IFs) of eukaryotic cells is still a matter of conjecture. On the basis of immunofluorescence and electron microscopic observations, they appear to play a cytoskeletal role in that they stabilize cellular structure and organize the distribution and interactions of intracellular organelles and components. The expression of a large number of cell type-specific and developmentally regulated subunit proteins is believed to provide multicellular organisms with different IF systems capable of differential interactions with the various substructures and components of their multiple, differentiated cells. However, the destruction of distinct IF systems by manipulation of cultured cells or by knock-out mutation of IF subunit proteins in transgenic mice exerts relatively little influence on cellular morphology and physiology and on development of mutant animals.

In order to rationalize this dilemma, the cytoskeletal concept of IF function has been extended to purport that cytoplasmic (c) IFs and their subunit proteins also play fundamental roles in gene regulation. It is based on the *in vitro* capacity of cIF(protein)s to interact with guanine-rich, single-stranded DNA, supercoiled DNA and histones, as well as on their close structural relatedness to gene-regulatory DNA-binding and nuclear matrix proteins. Since cIF proteins do not possess classical nuclear localization signals, it is proposed that cIFs directly penetrate the double nuclear membrane, exploiting the amphiphilic, membrane-active character of their subunit proteins. Since they can establish metastable multisite contacts with nuclear matrix structures and/or chromatin areas containing highly repetitive DNA sequence elements at the nuclear periphery, they are supposed to participate in chromosome distribution and chromatin organization in interphase nuclei of differentiated cells. Owing to their different DNA-binding specificities, the various cIF systems may in this way specify different chromatin organizations and thus the expression of distinct sets of cell- or tissue-specific proteins. In support of this, different type III IFs have been shown to preferentially interact with guanine-rich, highly repetitive, double-stranded fragments of total genomic DNA, including chromosomal telomere sequences. Surprisingly, they also bound AT-rich, centromeric satellite DNA sequences with high efficiency. Since most of the affinity-isolated, non-telomeric and -centromeric DNA fragments contain regulatory elements that are normally located in 5′/3′-flanking and intron regions of genes, cIFs may activate gene expression or repress it as the result of telomeric and centromeric position effects. However, the nucleotide sequences of the cIF-bound, genomic DNA fragments also predict the involvement of cIF(protein)s in recombination and hence in evolutionary processes. Based on these observations, the initially observed minor effects of cIF protein knock-out

mutations on the phenotype of transgenic mice may be interpreted as a redundancy phenomenon operating at the levels of the cytoskeleton and gene expression, whereas the capacity of the mutated animals to adapt to new environments via recombination processes may be severely disturbed and, as such, perceivable only after many generations of less favorable living conditions.

ALTHOUGH EXTENSIVE RESEARCH has been performed during the last 25 years on intermediate filaments (IFs), their biological role in eukaryotic cells is still largely unknown. On the basis of their cytoplasmic distribution, high mechanical stability, relatively low dynamics and association with a multitude of cellular components and substructures, they are generally believed to play a cytoskeletal role in that they participate, in cooperation with microfilaments and microtubules, in the formation and stabilization of cell structure and the spatial-temporal organization of the manifold physiological processes making up the life cycle of eukaryotic cells. These activities are documented by a host of immunofluorescence and immunoelectron microscopic observations made under both physiological and pathological conditions (for reviews, see 1–8). The fragility of epithelial cells as a result of mutation-induced assembly incompetence of cytokeratins is rated as particular support for the cytoskeletal concept of IF function (9, 10). In addition, the protein subunit composition of IFs of a large variety of cell- or tissue-specific subunit proteins, which during embryogenesis of vertebrate organisms are expressed following a distinct, genetically determined time schedule, seems to ascribe to them important functions in differentiation processes. Judging from the high degree of conservation of the individual subunit proteins of IFs during evolution, all of these functions appear to be of great importance to the morphology, physiology and development of cells, tissues and organisms. Intriguingly, despite their heterogeneity in subunit composition, IFs are of an astonishingly uniform, microscopic appearance, implying that, dependent on IF type, they fulfil cell type-specific functions, whereas, owing to their uniform construction principle, they play more fundamental roles in cellular activity. While some cell-specific activities have been detected for some IF types, common functions have not yet been disclosed for all of them, if one refrains from complete acceptance of their hypothetical, rather vaguely defined cytoskeletal role in the cytoplasm.

However, there are also a number of observations which cast serious doubt or impose complications on this merely cytoskeletal role of IFs. For instance, microinjection of IF-specific antibodies into cultured cells leads to a collapse of the cytoplasmically extended IF networks onto the nucleus without any significant effect on the structure and performance of the microinjected cells (11, 12). Moreover, a small collection of permanent cell lines can exist without any cytoplasmic (c)IF proteins (13–16), an observation which also applies to the cells of the inner cell mass of the blastocyst stage of mammalian embryogenesis (17, 18). Since in both cases the cells are undifferentiated, the observed lack of cIFs provides indirect support for their involvement in differentiation processes. Yet, even total knock-out of individual cIF protein genes in transgenic mice exerted little influence on the phenotype of the mutated animals (19, 20). These results are especially difficult to interpret since they question not only the cytoskeletal role of cIF proteins in particular but also their essentiality for cellular activities and developmental processes in general. There appears to be a contradiction between the high degree of evolutionary conservation of the different cIF proteins and their apparent dispensability for the manifestation of life of cells, tissues and whole organisms. Functional redundancy among cIFs and/or their subunit proteins on the

one hand and other cytoskeletal elements and/or differentiation-specific cell components on the other might provide an explanation for this discrepancy.

These brief, introductory remarks on IF proteins clearly show that a detailed understanding of their biological role is still a long way off. Since it is doubtful that the most promising means to reach this goal, the production and study of cIF protein-deficient transgenic animals, will yield sufficient and satisfactory information in the foreseeable future, the problem has to be tackled in the meantime on less sophisticated grounds. It is to be expected that characterization of the interaction of cIFs and their subunit proteins with well-defined cellular components and substructures in cell-free systems will allow a faster and more direct insight into the structural-functional relationships between cIF(protein)s and other cellular constituents *in vivo*. In the following sections, the structural and functional properties of pure cIF proteins and their filaments will be compared with related properties of other cellular components and the detected parallels will be used to advance a hypothesis on the cellular function of cIF(protein)s that is complementary to their cytoskeletal role.

Structure of IF proteins and their filaments

IF proteins belong to an extensive multigene family with approximately 40 different members which can be grouped into 6 different types on the basis of tissue-specific expression, sequence homologies, and gene organization: cytokeratins characteristic of epithelial cells (type I and II); mesenchymal vimentin, muscle-specific desmin, astroglial glial fibrillary acidic protein (GFAP), peripherin typical of neurons of the peripheral nerve system (type III); neurofilament proteins typical of neurons of the central and peripheral nerve system (type IV); lamins A, B, C constituting the nuclear lamina in all cells (type V); nestin characteristic of neuroepithelial stem cells (type VI) (for reviews, see 4, 8, 21). All cIF proteins possess a central, α-helical rod domain some 310 amino acid residues in length, which is subdivided at conserved sites by short, non-α-helical linker sequences into the subhelices IA, IB, IIA and IIB (Figure 1). The well-conserved rod domains are flanked by structurally highly variable, non-α-helical polypeptide regions, the N-terminal head and C-terminal tail domains. The major structural and chemical differences existing between the various cIF proteins are localized within these terminal sequences. The nuclear lamins are distinguished from their cytoplasmic relatives by a 6 heptad longer IB subhelix, relatively short head regions and long tail pieces which carry both a nuclear localization signal and at the very end an isoprenylation site. cIF proteins do not possess a nuclear localization signal and are therefore primarily restricted to the extranuclear compartment in interphase cells (4, 8, 21).

Owing to their strong tendency to form coiled-coil rope structures, the rod domains of two IF protein molecules wind around each other in axial register and parallel alignment with the formation of a dimer as the first intermediate in the IF assembly process. In a second step, two such dimers interact laterally in a staggered and antiparallel fashion with the formation of a tetramer, so that pairs of N-terminal head domains become situated on opposite ends of the oligomer (8, 21, 22). At this point, it is pertinent to mention that whereas cytokeratins I and II form obligatory heterodimers (8, 21, 22), the other cIF proteins can only produce homodimers, two of which, however, are able to assemble into either homo- or heterotetramers (23). The tetramers, also referred to as protofilaments, represent the actual

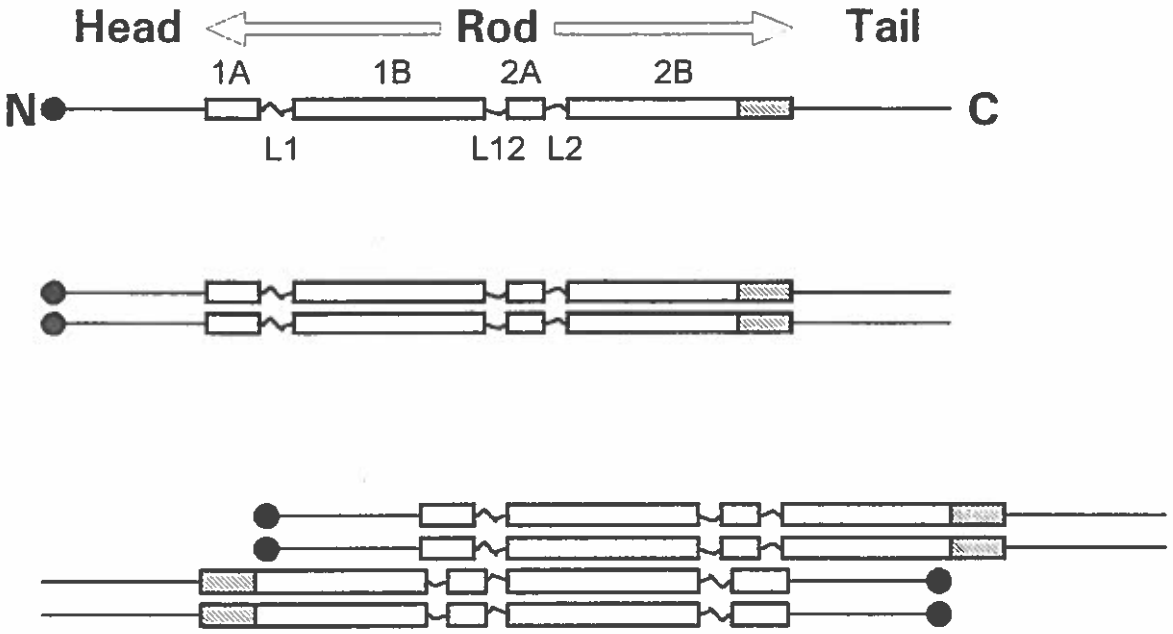


FIGURE 1. Oligomerization potential of cIF proteins. Tetramerization is a two-step process in which, in a first step, the rod domains of two parallel-oriented cIF protein molecules wind around each other in exact axial register and, in a second step, two dimers align in antiparallel and staggered fashion with the formation of a tetrameric protofilament. The non-α-helical head and tail domains are indicated by N (and/or by a closed circle at the end of the line) and C, respectively. The α-helical segments within the rod domain are indicated by the boxes and the non-α-helical linkers by the curved lines. The pitch of the α-helix reverses within helical segment 2B, as indicated by the diagonal shading. The actual lengths of the head and tail domains of the individual cIF proteins are quite variable. The tetramers achieve rotational symmetry via antiparallel alignment of two parallel-oriented dimers. Higher oligomerization entails further coiled-coil interactions in a longitudinally staggered fashion with the N- and C-terminal domains becoming largely exposed on the surface of the oligomers. Ideally structured, mature cIFs show 8 tetrameric coiled-coils in cross-section (24).

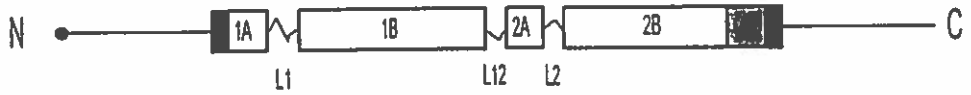
building blocks of the cIFs. While at low salt concentration the cIF proteins exist predominantly in their tetrameric forms, at physiological ionic strength the protofilaments polymerize in an as yet not well characterized assembly process into 10 nm filaments (8, 21, 22, 24). The 10 nm filaments, irrespective of subunit composition, are essentially morphologically indistinguishable. In the scope of the cellular function of cIFs, it suffices to state that the filament body proper is almost exclusively made up of the α-helical rod domains of the constituent subunit proteins and that the non-α-helical, N-terminal head and C-terminal tail regions are largely exposed on the filament surface in a probably helical arrangement. In this way, the structurally highly variable end domains endow the filament backbone with different surface properties or chemical reactivities and thus specify the functions of cIFs during development and differentiation (25).

Although the cIF assembly process is driven mainly by the lateral, largely hydrophobic associations of the α-helical rod domains of the constituent subunit proteins, the N-terminal head domains also actively contribute to filament formation and stability (for a review, see 26) (Figure 2). Truncation of the N-terminus by limited proteolysis causes assembly incompetence of the cIF proteins (27, 28) or, if preformed cIFs are treated with proteinases,

non-α-helical amino-terminal head domain (structurally variable)

α-helical rod domain (structurally conserved) (~310 amino acids)

non-α-helical carboxy-terminal tail domain (structurally variable)



Role in:
filament assembly,
interaction with
rod domain

Role in:
filament assembly,
self-self interaction and
interaction with head domain

Role in:
control of lateral
filament interaction

Posttranslational modification:
phosphorylation;
O-glycosylation;
ADP-ribosylation;
arginine deimination;
proteolysis (Ca^{2+}-dependent, retroviral)

Posttranslational modification:
phosphorylation;
proteolysis (retroviral)

Interaction with:
ankyrin;
nucleic acids;
anionic lipid bilayers;
other subcellular
structures

Interaction with:
core histones;
neutral lipids;
α-helical polypeptides?

Interaction with:
lamin B;
other subcellular
structures

FIGURE 2. Multi-functionality of multi-domain cIF proteins. The diagrammatic representation of a cIF protein monomer shows its consensus multi-domain structure consisting of a subdivided, α-helical rod domain flanked by non-α-helical head (N) and tail (C) domains. Below the individual domains, their functions in filament assembly, posttranslational modification and interaction with other cellular components are listed. The black boxes at both ends of the rod domain indicate the most conserved polypeptide stretches of the cIF proteins. Certain amino acid exchanges in these regions cause assembly incompetence of cIF proteins (34–36).

disassembly of the filaments (29). The shortened proteins can still form dimers and tetramers on their own or a series of heterooligomers in combination with intact protein molecules (23, 28). cIFs can tolerate a limited number of N-terminally truncated subunit proteins (28, 30). Removal of the C-terminus from the cIF proteins has no significant effect on filament assembly and stability (28, 31).

Since the head pieces of at least type III IF proteins show high affinities for the end regions of the α-helical rod domains (32, 33) (Figure 2), they might function in the correct lateral and staggered alignment of the protofilaments during filament polymerization and in

filament stabilization. Point mutations in the highly conserved rod ends severely affect the assembly competence of cIF proteins (34–36). Although such mutations modify the contact zones between the α-helical cores of the individual cIF building blocks, they also might weaken the interaction of the end regions of the rods with distinct sites on the N-terminal head domains. It is therefore likely that, based on this affinity, the N-terminal head domains have the potential to regulate filament formation and stability and thus cIF dynamics. Interestingly, the N-terminal head pieces are highly susceptible to posttranslational modification, such as phosphorylation, O-glycosylation, ADP-ribosylation and Ca^{2+}-dependent proteolysis and deimination of arginine residues (for references, see 37) (Figure 2). These reactions are known to cause labilization and reorganization of cIFs, presumably due to loosening of the interaction of the N-terminal head domains with the filament body. Another important effect of such processes is that the head pieces become available to interactions with other cellular components and substructures, whereby the modifications might have the additional advantage of increasing the affinity of the head pieces for their cellular targets. Thus, the effect of limited posttranslational modification may be the activation of the cIFs.

In vitro reactivities of IF proteins and their filaments

IF proteins are multi-domain proteins and should therefore be multi-functional (Figure 2). Their most obvious, but not necessarily most important function is their polymerization into IFs. This process involves their α-helical rod domains and the non-α-helical, N-terminal head regions, whereas their C-terminal tail regions are seemingly dispensable. The tail pieces appear to be primarily involved in controlling lateral interactions between mature cIFs (38) (Figure 2). In addition, they possess an oligopeptide region with high affinity for the lamin B component of the nuclear lamina (see also below) (39) (Figure 2). The interaction of lamin B with cIF proteins was postulated to create initiation sites for cIF assembly at the nuclear envelope, which then proceeds in a vectorial fashion through the cytoplasm to be terminated at the plasma membrane via interaction of the N-terminal head regions with the ankyrin component of the plasma membrane-associated cytoskeleton (40) (Figure 2). Otherwise, the C-terminal tail domains do not show outstanding sequence characteristics that could be responsible for the interaction of cIFs with other partners, except for those of the neurofilament proteins, which can be of enormous size and richness in glutamic acid residues. In their phosphorylated forms, they are involved in the formation of cross-bridges between individual neurofilaments (41, 42), an activity which in neuronal axons is exploited to maintain maximal distance between parallel aligned neurofilaments and to determine axon caliber (43).

a) Interaction of IF proteins with single-stranded nucleic acids

In comparison to the C-terminal tail regions, the N-terminal head regions are significantly more reactive, as can be judged from their high susceptibility to post-translational modification (Figure 2). At first glance, their wealth in arginine residues and paucity in acidic amino acid residues is most striking (44). Because of their high net positive charge, they should be capable of interacting with polyanionic compounds and molecular assemblies. Indeed, all cIF proteins tested, like vimentin, desmin, GFAP, the neurofilament proteins and some cytokeratins, prove to be excellent nucleic acid-binding proteins (for references, see 45, 46). In their protofilamentous form, they react preferentially with guanine-rich single-

stranded nucleic acids, with DNAs being somewhat better bound than RNAs. The isolated N-terminus of vimentin exhibits the same binding characteristics as intact vimentin, demonstrating that it represents the actual nucleic acid-binding domain (Figure 2). The same holds true for the N-termini of desmin and GFAP. The relative affinities of vimentin for 75 synthetic and naturally occurring DNAs and RNAs of different base composition and secondary structure differ by more than 2.5 orders of magnitude (47, 48). These differences clearly show that the interactions observed do not solely depend on ionic bonds between the multiple arginine residues of the N-terminal head domain of vimentin and the sugar phosphate backbones of the various nucleic acids and that they therefore cannot be of a totally unspecific nature. On the basis of theoretical studies on protein-nucleic acid interactions, it is probable that the arginine residues of the N-terminus of vimentin form stable hydrogen-bonded complexes with the guanine and cytosine bases of the polynucleotides (49).

It could further be shown that the N-terminus of vimentin makes additional use of a number of aromatic amino acid residues, mostly tyrosines, in its binding to single-stranded nucleic acids. A comparison of the N-terminus of vimentin with the nucleic acid-binding regions of two prokaryotic single-stranded DNA-binding proteins, the gene 5 protein of bacteriophage fd and the gene 32 protein of bacteriophage T4, reveals a very similar, rather periodic distribution of tyrosine residues in all 3 polypeptides. As in the case of these phage proteins, treatment of the isolated N-terminus of vimentin with tetranitromethane results in the nitration of nearly all tyrosine residues, concomitant with oligomerization of the polypeptide through cross-linking of different molecules via their tyrosine residues. These nitration and oligomerization reactions are almost completely suppressed in the presence of single-stranded fd DNA or ribosomal RNA, indicating that the tyrosine residues are protected from modification, very likely through intercalation of the aromatic side groups between the bases of the nucleic acids. This supposition was substantiated by UV- and fluorescence spectroscopic measurements (45).

The parallels between the N-terminus of vimentin and the gene 5 protein of bacteriophage fd could be extended by the generation of very similar secondary structure profiles for both proteins when the secondary structure prediction rules of Chou and Fasman were applied. Moreover, the N-terminal polypeptide produces complexes with single-stranded fd DNA which in electron microscopy are comparable to those obtained with gene 5 protein (50). Most interesting, however, is the fact that a partial amino acid sequence of the N-terminus of vimentin can be folded into nearly the same, homologous, antiparallel β-sheet structure which in the case of the gene 5 protein is responsible for the binding of the protein to single-stranded fd DNA (51). This similarity represents a nice example of convergent evolution of a nucleic acid-binding region.

b) Interaction of IF proteins with supercoiled, double-stranded DNA

cIF proteins are also able to bind specifically to double-stranded DNA, provided that it is under superhelical strain (52). Sucrose gradient centrifugation of the reaction products obtained from various cIF proteins and a mixture of supercoiled, relaxed and linearized plasmid DNA at low ionic strength revealed that only the superhelical DNA variant increases its sedimentation coefficient with increasing quantities of cIF protein, whereas the other, non-superhelical forms of plasmid DNA maintain their original sedimentation behavior. It is quite possible that the superhelical tension in the plasmid DNA is dissipated by local

unwinding of the double strand with the production of denaturation bubbles of single-stranded DNA or by the formation of other stress-absorbing non-B DNA configurations, such as Z-, triplex- and cruciform-DNA, to which cIF proteins may show high affinity and through which they may initiate the complexation process. In any event, in electron microscopy, molecules of supercoiled plasmid DNA appear to be at least partially relaxed when they are totally and smoothly covered with vimentin. The introduction of a single-strand break into the supercoiled DNA completely abolishes complex formation with vimentin or other cIF proteins at limited protein concentrations. Interestingly, during longer incubation in the presence of oxygen, vimentin occasionally forms ill-defined, apparently non-filamentous aggregates to which molecules of supercoiled plasmid DNA bind at high density (52). The structures formed are electron microscopically strikingly similar to those of histone-depleted metaphase chromosomes in which a centrally located, elongated protein scaffold is surrounded by an immense mass of huge loops of superhelical, genomic DNA (53). These properties place cIF proteins in the same class as nuclear or chromosomal matrix proteins, and it should be recalled in this connection that cIF proteins are close relatives of the nuclear lamins.

c) Interaction of intact IFs with nucleic acids

The association of mature cIFs with nucleic acids is predicted to differ from that of tetrameric protofilaments because the N-terminal head domains of the subunit proteins, the actual nucleic acid-binding regions, are incorporated into the filament structure and therefore not readily available for interactions with other components. In addition, the higher ionic strength conditions necessary for filament stabilization exert a weakening effect on the binding reactions. Nonetheless, nucleic acids are generally capable of also binding to intact cIFs in that they release N-terminal head domains from the filament core (46). Actually, there is a competition for the N-terminal head domains between the nucleic acids and protein regions of the α-helical rod domains of the filament body, to the effect that polynucleotides with a high affinity for cIF proteins cause fragmentation or even total disassembly of the cIFs (Figure 2). Particularly single-stranded DNAs of high guanine content are distinguished by such potentials (46). On the other hand, linear, double-stranded DNA molecules with their lower affinity for cIF proteins bind to the filaments without significantly affecting their structural integrity (46). The binding of nucleic acids and other negatively charged macromolecular components to cIFs is principally possible because the filaments can tolerate the occupation of a limited number of their N-terminal head domains without forfeiting their structural stability (28, 30).

Double-stranded DNAs can compensate for their generally weak affinities for cIFs by employing greater molecular lengths. Whereas at physiological ionic strength single-stranded nucleic acids tend to adopt a compact random coil structure, double-stranded DNAs maintain a flexible, extended configuration. In this way, they can bring consecutive molecular regions in register with a succession of N-terminal head domains on the filament surface, thus stabilizing their binding to cIFs through multi-site associations. Since the reactivity of double-stranded DNAs with cIFs also depends on their base composition —GC-rich DNAs react considerably better than AT-rich DNAs (46)—it must be assumed that natural, double-stranded DNA fragments interact with cIFs in a rather non-uniform fashion along their molecular extension, employing only their sequence elements of highest

binding activity. On the basis of these considerations, it is predicted that cIFs should show high affinities for repetitive, guanine-rich, double-stranded DNA sequences (see below).

d) Interaction of IF proteins and their filaments with lipids

Lipid membranes represent further biologically important macromolecular structures with strong potentials to interact with cIFs and their subunit proteins because of their often net negative surface charge. Vimentin filaments enter into intimate relationships with vesicles prepared from total cellular lipid, which they frequently appear to pierce (54). Employing liposomes consisting of anionic phospholipids, the observed associations could indeed be demonstrated to primarily depend on electrostatic interactions between the negatively charged lipid bilayers and the positively charged N-terminal head domains exposed on the surface of the filaments (55) (Figure 2). Liposomes prepared from phospholipids with electrically neutral head groups do not form stable complexes with preformed vimentin filaments. Since the N-terminal head domains are also essential for filament stability, their occupation by polyanionic phospholipid bilayers leads to destabilization and, in extreme cases, disassembly of vimentin filaments (Figure 2). Conversely, such lipid material prevents filament formation from tetrameric protofilaments (55). The various anionic phospholipid species show different activities in affecting filament formation and stability, phosphatidylinositol and its phosphorylation products, PIP and PIP_2, being the most active ones among the lipids tested. On a molar basis, these compounds are more effective than the ionic detergent SDS in disrupting cIF structure. Acting on vimentin protofilaments and IFs, phosphatidylinositol vesicles lose their integrity due to complexation of the constituent lipid molecules by vimentin molecules (55).

A more detailed analysis of the mechanism governing the reaction between vimentin and its filaments with polyanionic lipid vesicles has shown that the N-terminal head domain of the cIF protein not only initiates the reaction via electrostatic interaction with the negatively charged liposome surface, but also extends it via contacts with the hydrophobic interior of the lipid bilayer. UV illumination of complexes obtained from vimentin and negatively charged liposomes carrying photoactivatable groups or compounds in the center of the lipid bilayer causes labeling of the hydrophilic N-terminal head domain rather than of the hydrophobic α-helical rod domain of the cIF protein (56). Obviously, the initial electrostatic interaction induces massive molecular rearrangements in the lipid bilayer, which bring the N-terminal head domain in close contact with the hydrophobic fatty acid chains of the phospholipid molecules. Such rearrangements appear to be the cause of the leakage of polyanionic liposomes upon contact with vimentin and its filaments (57) and of the production of large membrane fusion products when the protein material is allowed to react with a large excess of anionic lipids (55). Nevertheless, it is likely that the α-helical rod domain of vimentin also participates in these reactions since it exhibits high affinities for neutral lipids, such as di- and triacylglycrol, cholesterol, cholesterol esters etc. (58) (Figure 2). The insertion of such neutral lipids into the lipid bilayer of negatively charged liposomes also increases the reactivity of the vesicles with vimentin and its filaments (58).

With respect to the membrane-active character of vimentin, it is noteworthy that the structure of its N-terminal head domain closely resembles the structures of the usually arginine-rich, amphiphilic signal peptides of mitochondrial precursor proteins (59). After their recognition by protein receptors on the mitochondrial surface (60), they mediate in a

phospholipid- and electrical potential-dependent manner the transport of nuclear-encoded protein molecules into the interior of the organelle (61 and references therein). During this transport process, the leader sequence is removed by a specific, mitochondrial proteinase and the proteins are targeted to their final destination sites. In addition to vimentin, other cIF proteins, particularly cytokeratin K8 (62), carry N-terminal head domains with properties similar to those of mitochondrial leader sequences. Interestingly enough, 20 and 22 KDa breakdown products of cytokeratin K8 were recently detected to be tightly associated with the inner mitochondrial membrane in hepatocytes of glucagon-treated rats (63). As discussed below, these parallels between the N-terminal head domains of cIF proteins and the signal sequences of mitochondrial precursor proteins serve as the basis for a potential mechanism governing the penetration of nucleus-associated cIFs through the double nuclear membrane.

e) Interaction of IF proteins with histones

With respect to a possible, general function of cIF proteins in eukaryotic cells, it is of particular relevance that their capacity to interact with DNA is complemented by their potential to associate with histones (64). Although cIF proteins bind all 5 major histone species, the core histones H2A, H2B, H3 and H4 and the linker histone H1, they prefer the core histones as reaction partners. This is clearly demonstrated when a constant amount of cIF protein is titrated at physiological ionic strength with a natural mixture of all 5 histone species. Already a small quantity of total histones is capable of transferring all of the cIF proteins into sedimentable complexes containing the various histone species, including histone H1, in nearly equal amounts. However, in the presence of slightly larger amounts of histones, the linker histone H1 quickly and completely disappears from the complexes, followed by the core histones H2A and H2B, so that in the presence of a large excess of histone mixture the association products contain, in addition to cIF protein, essentially only the arginine-rich core histones H3 and H4. This result is surprising insofar as the cIF proteins are acidic and the histones basic proteins and therefore largely electrostatic interactions of possibly little physiological relevance should be expected. Yet, the fact that the most basic histone species, the linker histone H1, is rejected by the cIF proteins as long as a slight excess of core histones is present argues for a rather specific, non-ionic binding component in addition to electrostatic interactions. Complex formation occurs exclusively through the α-helical rod domains of the cIF proteins (Figure 2) and the central, globular regions of the histones. Aside from the specificity of the cIF protein-histone interaction, its stoichiometry is of great importance: each tetrameric protofilament binds 16 core histone molecules, i.e. 2 nucleosome equivalents, irrespective of the composition of the histone moiety.

From these results, close parallels between the binding of core histones to double-stranded DNA and cIF proteins immediately become apparent. In nucleosomes, 2 loops of double-stranded DNA wind around a protein core consisting of 8 core histone molecules (65); the same number of core histone molecules interact with a cIF protein dimer. While in both cases the arginine-rich core histones H3 and H4 play a central role, the core histones H2A and H2B appear to occupy peripheral positions in both complexes and to be of secondary importance. On the basis of this parallelism, it is conceivable that under appropriate conditions core histones are exchangeable between both reaction partners, double-stranded DNA and cIF proteins. In other words, if cIF protein aggregates should happen to contact chromatin it should principally be possible for them, by employing their α-helical

rod domains, to dissociate core histones from nucleosomes and thus to liberate superhelical DNA; this could then be bound, in a concerted reaction, by the neighboring N-terminal head domains. These potentials would make cIF proteins comparable to regulatory DNA-binding proteins or transcription factors, a limited number of which, in fact, have recently been shown to disrupt nucleosomal structure (66 and references therein).

Comparison of IF proteins with transcription factors and nuclear matrix proteins

The comparison of cIF proteins with transcription factors is not as far-fetched as it might appear at first glance, since there are in fact many structural and functional features common to both protein classes (Table I). First and foremost, their possession of a mostly α-helical dimerization motif capable of parallel coiled-coil formation and flanked by a basic DNA-binding region often on the N-terminal side has to be emphasized. While the parallel, in register alignment of two protein molecules permits the recognition of dyad-symmetric DNA sequence motifs, the neighboring DNA-binding regions impart sequence specificity to these interactions (67, 68). In many cases, the highly unstructured DNA-binding regions assume their active configurations only after having contacted their corresponding DNA target sites (69). Similar to a number of regulatory DNA-binding proteins, such as prokaryotic repressor proteins (70) or the eukaryotic TATA box-binding factor TFIID (71), vimentin makes use of an antiparallel β-sheet structure in binding to DNA (45). Moreover, many transcription factors act not only as dimers but also as tetramers or higher oligomers, or even as polymers (72–74). Because in their oligo- and polymeric forms they expose multiple DNA-binding regions on their surface, they are capable of interconnecting distant, cis-acting DNA sequence elements, for instance enhancer and promoter sequences, through a protein bridge with looping out of the intervening DNA sequence (75). The stability of the complexes is enhanced significantly by DNA supercoiling (76). cIF proteins would ideally fulfil all of these functions owing to their oligo- and polymerization potential (Figure 1) and their characteristic DNA-binding activities. One special transcription factor, the zeste gene product of Drosophila, has been shown to lose its regulatory potential upon nullification of its polymerization competence by the introduction of point mutations in its α-helical dimerization motif. Lower oligomers of the zeste gene product are incapable of engaging in multi-site interactions and interconnecting a series of consensus DNA sequences occurring in distant regions of the genome (77). cIF proteins also respond sensitively to point mutations in the highly conserved end regions of their α-helical rod domains (34); in the case of cytokeratins, such assembly-incompetent mutant proteins have been demonstrated to be the cause of various genetic skin diseases (35, 36). The oligomerization potential of transcription factors also provides the structural basis for the production of dominant-negative mutant factors in that it allows the heterooligomerization of intact and truncated factor molecules deprived of their DNA-binding regions (67, 68). Such oligomers have lost their specific DNA-binding activity and can therefore function as antagonists of differentiation programs (78). Likewise, cIF proteins which have been deprived of their N-terminal nucleic acid-binding sites can still assemble into heterooligomers with intact protein molecules (23, 28, 30). These structurally modified oligomers definitely exhibit drastically altered nucleic acid-binding properties. Last, not least, many regulatory DNA-binding proteins have to

TABLE I. Comparison of the structural-functional relationships of transcription factors and cIF proteins. The comparison is largely limited to those transcription factors whose dimerization potentials are based on α-helical polypeptide regions capable of parallel coiled-coil formation.

Structural-functional features	Transcription factors	IF proteins
Cell or tissue-specific expression	ever-increasing number of cell-specific proteins	more than 40 different subunit proteins; development and tissue-specific expression
Dimerization motif	parallel, in axial register alignment of mostly α-helical polypeptide regions; coiled-coil formation (leucine zipper ZIP and helix-loop-helix HLH motifs)	parallel, in axial register alignment of central, α-helical rod domain; coiled-coil formation
Basic DNA-binding motif	disordered polypeptide region flanking the dimerization motif often on the N-terminal side (bZIP, bHLH, bHLH-ZIP proteins)	unstructured, N-terminal head domain
Oligomerization potential	dimerization of dimers and stacking of tetramers (GC box-binding factor Sp1 and lac repressor)	formation of symmetrical protofilaments by antiparallel alignment of two dimers; formation of higher oligomers
Heterooligomerization potential; dominant negative mutants	formation of hetero-dimers and tetramers from intact and truncated proteins missing the DNA-binding region (Id protein of bHLH factor family)	formation of hetero-dimers and tetramers from intact and N-terminally truncated proteins
Polymerization potential	formation of multivalent, linear aggregates (zeste gene product, retinoblastoma suppressor protein)	formation of IFs with multiple N-terminal head domains on the filament surface
DNA-looping potential	tetramers with pairs of DNA-binding regions at opposite ends; higher oligomers and polymers (Spl factor, lac repressor)	symmetrical tetramers with pairs of the DNA-binding, N-terminal head domains at opposite ends; higher oligomers and polymers
Histone-binding potential	binding domain unknown (GAGA and GAL 4 factor)	central, α-helical rod domain
Post-translational modification	activation and/or nuclear transport following post-translational modification (phosphorylation, O-glycosylation, etc.)	extensive modification potential in N-terminal head domain (phosphorylation, O-glycosylation, etc.)

overcome the constraints imposed on nuclear chromatin by the incorporation of cis-acting DNA sequence elements into nucleosomes. There are indications that some protein factors are capable of suspending nucleosomal repression by disruption of nucleosomes via at least partial removal of their histone moieties (66). cIF proteins would easily fit in this group of protein factors because of the strong histone-binding activity of their α-helical rod domains (64). Based on these parallels between cIF proteins and transcription factors, a model has been developed regarding the potential role of cIF protein oligomers in transcription initiation and elongation (37, 79).

In addition to their relatedness to transcription factors, cIF proteins also share structural and functional properties with nuclear matrix proteins (for references, see 37). The closest nuclear relatives are, of course, the nuclear lamins which themselves belong to the family of IF proteins. Underneath and in direct association with the inner nuclear membrane, they constitute the filament network of the nuclear lamina, a structure believed to be involved in the stabilization of the nucleus and in the organization of interphase chromatin. In the latter function, the lamins probably make use of their DNA-binding activities which are, however, less prominent then those of the cIF proteins, at least in *in vitro* assays (80). In any event, UV irradiation of intact cells covalently crosslinked a variety of DNA fragments containing repetitive sequences homologous to introns and/or flanking regions of different genes to the nuclear lamina (81). The lamin B component of the nuclear lamina was also shown to have MAR-binding properties (82). The nuclear mitotic apparatus (NuMA) protein is another nuclear protein possessing a central, α-helical rod domain capable of coiled-coil formation and flanked by a basic, non-α-helical terminal polypeptide region. It associates with splicing complexes (83) and probably provides a bridge between RNA processing and the nucleoskeleton, particularly its core filament complement. These core filaments are morphologically indistinguishable from cIFs (84–86). Their protein composition is still unknown, yet their 10 nm diameter and 23 nm periodicity also seen in their cytoplasmic relatives suggest that they are composed of subunit proteins structurally very similar to cIF proteins. The core filaments probably represent the basic karyoskeletal network with which most of the intranuclear components and activities are associated.

Interaction of IFs and their subunit proteins with the nucleus

The capacity of cIF proteins to bind to a large variety of different configurations of DNA and to histones, in conjunction with their structural similarity to certain transcription factors and nuclear matrix proteins, suggests that they not only fulfil cytoskeletal functions in the cytoplasm but that they are also involved in intranuclear processes. Of course, to be able to operate within the nucleus, they have to surmount the barrier of the double nuclear membrane and to establish contacts with intranuclear structures. This is a prerequisite that at first glance does not seem to be satisfiable because of the lack of conventional nuclear localization signals on these proteins. Nevertheless, cIFs are endowed with capacities which probably allow them to penetrate the nuclear envelope, circumventing the nuclear pore complexes. In gaining access to the nuclear interior, they apparently take advantage of their conspicuously high concentration in the immediate vicinity of the nucleus. In fibroblast cells, large perinuclear accumulations of vimentin filaments give the impression that they are in direct association with the nuclear surface. Indeed, in whole mount preparations of fibroblasts and other cell types, in which large parts of the nuclear membrane and nuclear

chromatin and ribonucleoprotein material have been extracted, the vimentin and other cIFs are visualized to contact and to intermingle with the nuclear lamina (6, 87, 88). Such associations are particularly clearly seen in stereo electron micrographs. Disregarding the possibility that the cIF systems have simply collapsed on the nucleus after extraction of the double nuclear membrane with nonionic detergent, these results indicate that under normal conditions the cIFs manage to penetrate the nuclear membrane barrier and to contact underlying nuclear matrix-chromatin complexes. Since in immunofluorescence microscopy the interior of the nucleus is normally free of cIF proteins, the filaments having entered the nucleus necessarily must be locally restricted to the nuclear periphery. This spatial limitation is logical given that the C-terminal tail domains of cIF proteins possess a short oligopeptide region with an affinity for the lamin B component of the nuclear lamina (89, 90). It is located in a highly repetitive arrangement on the filament surface and should anchor IFs to the nuclear lamina. The neighboring N-terminal head domains would remain unaffected and therefore still be available for interactions with nuclear matrix-chromatin complexes at the nuclear periphery. Experimental support for this scenario comes from the finding that in transfected cells cIF proteins deprived of their C-terminal tail domains also accumulate in the interior of the nucleus (31, 91, 92).

Assuming nucleus-associated cIFs in fact traverse the perinuclear cisternae bounded by the two nuclear membranes, the question arises how they achieve this when they cannot make use of nuclear translocation signals. One attractive possibility rests on the fact that the N-terminal head domains of cIF proteins are of an amphiphilic, membrane-active character (56, 62). Possibly recognized by receptor proteins on the outer nuclear membrane, the multitude of head domains arranged on the surface of the filaments might bring about in a cooperative effort dramatic perturbances in the structure of the nuclear envelope, thus paving the way for the filaments to enter the nucleus and to interact with their target sites. The overall process might be very similar to that operating in the translocation of nuclear-encoded proteins through the double mitochondrial membrane into mitochondria, which is also mediated by amphiphilic signal peptides (59, 61). Acting within bundles, individual filaments could take different parts in this process. While the peripheral filaments of a bundle open the perinuclear cisternae, the central filaments can pass through the opening while preserving their structural integrity. Indeed, in human breast carcinoma cells, bundles of cytokeratin filaments frequently hit the nucleus, disintegrate the nuclear envelope at the contact site and appear to interact through their central part with underlying nuclear matrix-chromatin complexes (unpublished results, see also 93).

Of course, there are other ways via which cIF proteins may gain access to the nuclear interior (for a discussion, see 37). The simplest way would be during mitosis when the cIFs are structurally labilized or even partially disassembled as a result of posttranslational modification of their N-terminal head domains. At the same time, the nuclear matrix and nuclear envelope are broken down and their posttranslationally modified constituents are released into the cytoplasm. Because of close structural relatedness, they may interact with activated cIF proteins and carry them into the newly-forming nucleus during the telophase. One such carrier protein could be lamin B (39, 40, 94). Even metaphase chromosomes themselves may function in this way since they have repeatedly been observed to be decorated with cIF protein material (95–97). However, also during interphase, posttranslationally activated cIF proteins may be loaded on karyophilic carrier proteins and transported piggy-back into the nucleus. Several transcription factors lacking nuclear localization

signals are targeted into the nucleus by such transport proteins (for references, see 79). Having once entered the nucleus, the cIF proteins must find their targets which appear to be localized at the nuclear periphery. Even then, they may depend on transport vehicles. In this respect, the finding may be of relevance that lamins are not exclusively confined to the nuclear periphery but also occur throughout the interior of the nucleus (98). Since the internal lamins relocate to the nuclear lamina with time, they may guide misdirected cIF protein molecules to their actual targets at the nuclear periphery. In any case, there is at least suggestive evidence that cIF proteins do occur within the nucleus. cIF proteins can be crosslinked to DNA in normal cells (99–101) and a vimentin epitope has been localized to chromatin structures (97). Furthermore, in interphase nuclei of nerve cells, cIFs impose constraints on chromatin motion (102) and agents altering its rate change gene expression (103). Yet of special relevance in the present context are the observations that C-terminally truncated (31, 91, 92) or ectopically expressed (104) cIF proteins accumulate as fibers or granules within the nucleus.

Taken together, these observations and considerations provide substantial support for the notion that there is no absolute barrier excluding cIF(protein)s from the nuclear interior. Their occurrence in the nucleus would not be exceptional since considerable quantities of other cytoskeletal proteins lacking nuclear localization signals, such as actin, tubulin, myosin and their associated proteins, have been found in the interphase nuclei of animal cells (37, 105; for further references, see therein).

Potential role of IFs and their subunit proteins in gene regulation

The intracellular distribution of cIF proteins with their preferential localization in the perinuclear area, their *in vitro* reactivities with nuclear constituents and their structural and functional similarities to regulatory DNA-binding proteins and nuclear matrix elements demarcate the scope of a concept for a general biological role of these proteins in gene regulation. It is postulated that nucleus-associated cIFs penetrate the perinuclear cisternae in order to contact nuclear matrix-chromatin complexes lying underneath the inner nuclear membrane. Attached to the nuclear lamina via the C-terminal tail domains of their constituent subunit proteins, they fix through their N-terminal head domains chromatin regions containing distinct DNA sequences at the utmost nuclear periphery. To achieve this, they take advantage of the histone-binding potential of the α-helical rod domains of their subunit proteins and of their capacity to interact with highly repetitive DNA sequence elements in a multi-site association process. A very essential aspect of this concept is that the cIF proteins operate as large aggregates, optimally in the form of their filaments, because only in this state is the local concentration of the N-terminal head domains high enough to effect the penetration of the double nuclear membrane and the localization of repetitive DNA sequence elements at the nuclear periphery in a cooperative effort. Any measure that destroys the aggregation potential of the cIF proteins will also cancel most of their nucleus-specific activities. In this way, the various cIF systems may participate, operating from the cytoplasm, in the distribution of chromosomes and organization of chromatin in interphase nuclei and thus in the regulation of DNA-based nuclear events, such as DNA replication, transcription, recombination etc. In addition, employing their chromatin-modifying capacity, cIFs having entered the nucleus may set the stage for the action of other, more generally

operating regulatory DNA-binding proteins. Hence, cIF proteins may be considered as commitment proteins or chromatin-modifying factors, for which the GAGA factor of Drosophila represents an illustrative example (106).

To have better control of the nuclear activities of cIF proteins, the cell probably employs its manifold posttranslational modification systems in conjunction with a variety of signal transduction pathways (for references, see 37). Physiological as well as developmental stimuli are transmitted to the perinuclear area and the nuclear envelope to stimulate their enzyme systems which in turn activate nucleus-associated cIFs. Actually, many of the posttranslational modification systems are located on or in the nucleus. It is certainly not accidental that most of the modifications, such as phosphorylation, O-glycosylation, ADP-ribosylation, Ca^{2+}-dependent proteolysis etc., concentrate on the N-terminal head domains of the cIF proteins, since these very likely represent the active principle in permeabilizing the nuclear membranes and in selecting DNA sequence elements to be fixed at the nuclear periphery. In their modified forms, they are not only partially released from the filament body and made available for reactions with other nuclear components but also rendered more specific in their reactivities with these constituents.

The present model also allows for a better understanding of the cell- or tissue-specific expression of cIF proteins. Owing to their different DNA-binding specificities, the various cIF systems may be functional in determining distinct states of chromatin organization and, as an immediate result of this, the expression of distinct sets of tissue-specific proteins and cellular activities.

With regard to the involvement of cIF systems in differentiation processes at the nuclear level, this concept is also in excellent agreement with the model concerning the molecular mechanism for the process of "reverse transformation" (107). This phenomenon encompasses the restoration of a normal, differentiated phenotype upon treatment of malignant cells with cyclic AMP. It includes as its most essential element the exposure of sets of cell- or tissue-specific genes in a transcriptionally active state in a rim around the nuclear periphery and the sequestration of other genes irrelevant for the respective differentiation state in the interior of the nucleus. As a consequence, the pattern of protein synthesis changes in a differentiation-specific manner. Based on these observations, normal differentiation is considered to require a different spectrum of genes to be exposed in each separate state of differentiation. It was further postulated, and experimentally partially substantiated, that the mechanism of transition between gene sequestration and exposure is controlled by the cyto- and karyomatrix in that their constituent filament systems, including cIFs (108), are attached to repetitive DNA sequence elements of specific chromosomal loci at the nuclear periphery. Organizational changes in these filament systems caused by stimulus-induced posttranslational modification would then provoke differentiation-specific changes in the pattern of gene sequestration and exposure.

Selective binding of specific DNA fragments to IFs *in vitro*

In order to confirm one of the predictions of the above model, that cIFs after having penetrated the perinuclear cisternae attach to chromatin regions containing repetitive DNA sequence elements, an attempt was undertaken to prove if cIFs indeed show a preference for repetitive sequences when interacting with total nuclear DNA. For this purpose, a mixture of genomic mouse DNA fragments was screened in an affinity binding assay with various

TABLE II. Summary of sequence types obtained by affinity binding of mouse genomic DNA fragments to *in vitro* reconstituted type III IFs.

Sequence type	Vimentin (77 fragments)	Desmin (118 fragments)	GFAP (74 fragments)
$(GT)_n$ perfect/imperfect	44% (34)	5% (6)	4% (3)
$(GA)_n$ perfect/imperfect	12% (9)	1% (1)	4% (3)
γ-satellite	17% (13)	38% (45)	12% (9)
minor satellite	0% (0)	11% (13)	5% (4)
LINE	1% (1)	26% (31)	28% (21)
SINE	0% (0)	0% (0)	8% (6)

in vitro reconstituted type III IFs. Nuclear DNA from Ehrlich ascites tumor cells was fragmented by ultrasonication, blunt-ended, ligated with polymerase chain reaction (PCR) primers and offered to *in vitro* assembled vimentin, desmin or GFAP filaments. The filament-DNA complexes were separated from unbound DNA by centrifugation, and the bound fragments were isolated and amplified by PCR. Thereafter, the amplified DNA fragments were subjected to 2 to 3 additional cycles of IF-affinity binding and PCR amplification, cloned and sequenced.

As expected, most of the fragments exhibiting high affinity for cIFs were rich in guanine and highly repetitive. Vimentin IFs (Table II) selected preferentially microsatellite DNA sequences mostly of the $(GT)_n$ and $(GA)_n$ type with perfect, imperfect as well as compound dinucleotide runs. Frequently, other purine-rich fragments of more complicated base sequence were recognized. A considerable number of fragments did not show particular sequence characteristics, except that they contained short guanine runs on both DNA strands in a rather homogeneous distribution. These fragments are structurally very similar to linear, extrachromosomal DNA molecules that have been isolated from the cytoplasm of transformed mouse cells and that are capable of immortalizing human lymphocytes (109). Telomere repeats were expected among the fragments selected by vimentin IFs on the basis of previous studies showing that vimentin binds with high affinity to the guanine-rich repeats of chromosome telomeres (110). Although such fragments bound to the filaments in the first binding step, they could not be amplified because of secondary structure problems and were therefore lost. Contrary to all expectation, a relatively large number of mouse major or γ-satellite DNA fragments were isolated. γ-satellite DNA is composed of a long tandem repeat of an AT-rich, 234 bp consensus sequence and occurs in the centromeres of all mouse chromosomes (111). Its efficient binding to vimentin IFs may be due to its bent structure which results from repeats of short adenine runs interrupted by relatively guanine-rich sequence elements (112). *In situ* hybridization employing mouse metaphase chromosomes indeed localized the isolated γ-satellite fragments to the centromeric region of the chromosomes.

In contrast to vimentin IFs, desmin IFs (Table II) selected microsatellite repeats of the $(GT)_n$ and $(GA)_n$ type infrequently, but reacted all the better with γ-satellite DNA. Additionally, they picked up minor satellite DNA fragments with considerable efficiency. These are derived from another long tandem repeat of an AT-rich, 120 bp consensus sequence occurring in the pericentromeric region of mouse chromosomes (113). Aside from frag-

ments rich in short guanine runs, some of which contained CpG islands, several minisatellite sequences were characteristically recognized by desmin IFs. Desmin IFs were also far superior to vimentin IFs in associating with LINE sequences, long interspersed nucleotide elements.

In general, GFAP IFs (Table II) were closer to desmin than to vimentin IFs in their DNA-binding characteristics. They were distinguished not only by their preferential binding of LINE sequences but also by their affinity for SINE sequences, short interspersed nucleotide elements, which so far have not yet been found to associate with vimentin and desmin IFs.

Although there remains statistical uncertainty due to the small sample sizes analyzed, this preliminary comparison of the DNA-binding activities of vimentin, desmin and GFAP IFs (Table II) already shows that the 3 filament systems are qualitatively different in their selection for specific DNA sequences from a mixture of genomic DNA fragments. The fact that they bound certain types of DNA fragments, like the $(GT)_n$ (114) and $(GA)_n$ (115) microsatellite or the γ-satellite DNA (111) sequences, with a much higher frequency than could theoretically be expected from their natural abundance in the mouse genome points to a considerable degree of specificity in the binding reactions. The results obtained may be rated as confirmation of the notion that the various cIF systems are involved in the differential organization of chromatin in the interphase nuclei of differentiated cells. So far, no true consensus nucleotide sequences, as are recognized by classical transcription factors, could be detected for any of the individual cIF proteins. Possibly, such sequences do not exist, because cIFs may recognize and bind to specific secondary or higher level structures based on a multitude of related sequences, thus allowing for gene regulation on a global scale.

In fulfilling such functions, the cIFs may, among other things, employ their affinities for the highly repetitive DNA sequence elements of the centromere and telomere regions of chromosomes. In fact, in addition to major and minor satellite DNA fragments, various other DNA sequences bound to the cIFs could be localized to the centromere region by *in situ* hybridization. In this context, it should be noted that in certain cell types centromeres and telomeres are preferentially localized to the nuclear periphery in cell type-specific, nonrandom patterns that relate to the differentiation state of the cells (116–118). Principally, other repeat elements of sufficient extension recognized by the cIFs might be metastably immobilized at the nuclear periphery by nucleus-associated cIFs. Moreover, since most of the DNA fragments selected by the cIFs also contain gene-regulatory sequences (so far only shown for fragments bound to vimentin IFs), cIFs might provoke gene silencing via translocation of these elements into the heterochromatic centromere and telomere regions of chromosomes. Making use of their DNA looping potentials, they could thus bring about centromeric and telomeric position effects. However, they may also effect gene activation by avoiding the centromere and telomere regions and contacting only gene-regulatory elements in certain chromatin areas.

Among the gene-regulatory elements detected in the cIF-bound genomic DNA fragments, the $(GT)_n$ and $(GA)_n$ microsatellites deserve some special consideration since they have been selected in such high proportions by vimentin IFs. Both microsatellites are highly but not randomly dispersed in eukaryotic genomes (114). They are thought to be involved in the regulation of gene expression because they occupy relatively conserved positions within non-coding, 5′/3′-flanking and intron regions of genes (119–125), can influence the

transcriptional activity of genes in plasmid constructs (123, 125, 126) and are components of nuclear matrix attachment regions (127). Their potential to undergo transitions from B- to Z- and triplex-DNA (122, 128), respectively, under negative superhelical tension and to be recognized in these configurational states by specific regulatory DNA-binding proteins (123, 125, 129) is certainly of physiological relevance in this respect. In addition, a FASTA search of the EMBL data bank revealed other control region sequences in the vimentin IF-specific DNA fragments, particularly a large number of perfect matches for a variety of known transcription factors. Finally, many of the fragments possessed inverted repeat DNA sequences capable of adopting cruciform structure in response to negative supercoiling. Such structures are also recognized by specific protein factors and act as regulatory signals in DNA transcription, recombination and replication (130). It appears from these findings that most of the non-centromeric DNA fragments bound to vimentin IFs contain gene control regions. In accord with this supposition, a meaningful open reading frame with regulatory elements in the 5′-flanking regions could be identified only in 1 among 75 sequenced fragments.

Judging from the repetitiveness and high guanine content of most of the cIF-bound DNA fragments, it may further be predicted that nucleus-associated cIF(protein)s are also, if not mainly, involved in recombination processes. $(GT)_n$ and $(GA)_n$ microsatellites have been proposed to be necessary but not sufficient components of recombination hotspots (114, 131, 132), and are present in synaptonemal complexes of pachytene chromosomes (133). The same seems to hold true for guanine-rich minisatellites (134). Since many of the fragments with short guanine runs on both DNA strands contain one or more repeats of the functional minimal sequence GGNNGG from the oncogene BCL-2 major chomosomal breakpoint (135), they also may represent hotspots of recombination. γ-satellite repeats have been shown to be a frequent site of integration of minisatellite-containing transgene DNA into the mouse genome and considered as sites of recombination between nonhomologous chromosomes (111).

Since the biological role of LINE/SINE sequences is poorly understood, there is only little room for speculation on the function of the association between such sequence elements and cIFs. In contrast to the tandem repeats of satellite DNAs, LINE/SINE elements are interspersed, repetitive DNA sequences. They are mobile, transposable elements (retrotransposons/retroposons) that amplify via reverse transcription of an RNA intermediate and are then inserted nonrandomly into new genomic sites, with all the consequences for gene regulation (137–139). Because the dispersal of LINE elements is thought to occur in the germline, it is not unexpected that their expression is particularly high in certain embryonal carcinoma and teratocarcinoma cell lines (140, 141). It is not known which proteins mediate the retroposition events. Should cIFs indeed participate in these insertional mutation processes and thus contribute to the plasticity of the genome, they might play an important role in the adaptation of natural populations to new environmental conditions.

Concluding remarks

In view of the tight association of cIFs with the nucleus, their capacities to perturb and penetrate the nuclear membrane and to interact specifically with elements of the nuclear matrix, single-stranded DNA, supercoiled DNA, distinct sequences of double-stranded

DNA as well as histones it seems likely that they are intimately involved in nuclear processes at the chromatin/DNA level. As cellular constituents with properties very similar to those of regulatory DNA-binding and nuclear matrix proteins, cIFs are postulated to participate, operating from the cytoplasm, in the organization of interphase chromatin and thus in gene regulation. Specifically, they may be involved in the control of gene expression in that they act as commitment or chromatin-modifying protein assemblies, preparing interphase chromatin for the binding of more generally operating gene-regulatory factors. Their penetration of the nuclear membrane and contact with underlying nuclear matrix/chromatin complexes is thought to be induced or modulated by their activation via posttranslational modification in response to physiological and developmental signals from intra- and extracellular space. This view is in no way contradictory to the cytoskeletal concept of cIF function; it would only extend it by purporting that cIFs as constituents of the cytoskeleton fulfil their functions not only in the cytoplasm and on the bordering nuclear envelope and cytoplasmic membrane but also in the periphery of the nuclear interior after penetration of the double nuclear membrane.

However, cIF(protein)s may play an even more important role in recombination and evolutionary processes. If they should indeed act as recombination-promoting factors, this would allow a better understanding of the so far minor effects of knock-out mutations of individual cIF proteins on the phenotype of transgenic mice, because the mutated animals would need many generations to manifest the genetic defects, particularly when they are kept under non-challenging environmental conditions. At the level of gene expression, such knock-out mutations may be compensated for by other regulatory DNA-binding factors that animal cells can produce in great number, whereas at the cytoskeletal level other filament systems with their associated proteins may take over the tasks of the missing cIFs. The knock-out of two or more cIF proteins at the same time will probably be lethal; if so, this would definitely corroborate the essentiality of this protein class as a whole for the development and physiology of animal organisms.

In order to find out whether or not cIF proteins indeed are charged with important functions in recombination and thus evolutionary processes, future studies will concentrate, among other things, on possible relationships between cIF proteins and extrachromosomal circular DNA. Such DNA is under superhelical tension and known to contain repetitive sequence elements similar to those strongly bound to cIFs (142). It can therefore be expected to have a particularly high affinity for cIF proteins. Since, on the other hand, extrachromosomal circular DNA appears to be the product of intrachromosomal recombination processes and to shuttle between the nucleus and the cytoplasm, it is reasonable to assume that it is constantly loaded with cIF proteins and that these proteins then catalyze the recombination processes. In this way, cIF proteins could not only exert an influence on cell-specific regulation of growth and gene expression and genome stability during development and aging but also, in the long run, allow natural populations to respond to environmental challenges.

My sincerest thanks are due to Dr. Robert L. Shoeman, Genrich Tolstonog, Dr. Xiao Wang, Dr. Constantin E. Vorgias, Dr. Georgios Perides and Dr. Siegfried Kühn for their experimental and intellectual contributions to the concept of cIF function outlined in the present article. I also thank Dr. R.L. Shoeman for his critical reading of the manuscript and Mrs. Lolita Horak for secretarial work.

References

1. Lazarides, E. (1980) *Nature* (London) *283:*249-256.
2. Traub, P. (1985) "Intermediate Filaments. A Review". Springer Verlag, Heidelberg.
3. Steinert, P.M. and Roop, D.R. (1988) *Annu. Rev. Biochem. 57:*593-625.
4. Bloemendal, H. and Pieper, F.R. (1989) *Biochim. Biophys. Acta 1007:*245-253.
5. Klymkowsky, M.W., Bachant, J.B. and Domingo, A. (1989) *Cell Motil. Cytoskel. 14:*309-331.
6. Carmo-Fonseca, M. and David-Ferreira, J.F. (1990) *Electron Microsc. Rev. 3:*115-141.
7. Skalli, O. and Goldman, R.D. (1991) *Cell Motil. Cytoskel. 19:*67-79.
8. Fuchs, E.K. and Weber, K. (1994) *Annu. Rev. Biochem. 63:*345-382.
9. Fuchs, E. (1994) *J. Cell. Biol. 125:*511-516.
10. McLean, W.H.I. and Lane, E.B. (1995) *Curr. Opinion Cell Biol. 7:*118-125.
11. Gawlitta, W., Osborn, M. and Weber, K. (1981) *Eur. J. Cell Biol. 26:*83-90.
12. Klymkowsky, M.W., Miller, R.H. and Lane, E.B. (1983) *J. Cell Biol. 96:*494-509.
13. Venetianer, A., Schiller, D.L., Magin, T. and Franke, W.W. (1983) *Nature* (London) *305:*730-733.
14. Giese, G. and Traub, P. (1986) *Eur. J. Cell Biol. 40:*266-274.
15. Hedberg, K.K. and Chen, L.B. (1986) *Exp. Cell Res. 163:*509-517.
16. Röser, K., Bohn, W., Giese, G. and Mannweiler, K. (1991) *Exp. Cell Res. 197:*200-206.
17. Jackson, B.W., Grund, C., Schmid, E., Bürki, K., Franke, W.W. and Illmensee, K. (1980) *Differentiation 17:*161-179.
18. Paulin, D., Babinet, C., Weber, K. and Osborn, M. (1980) *Exp. Cell Res. 130:*297-304.
19. Colucci-Guyon, E., Portier, M.-M., Dunia, I., Paulin, D., Pournin, S. and Babinet, C. (1994) *Cell 79:*679-694.
20. Gomi, H., Yokoyama, T., Fujimoto, K., Ikeda, T., Katoh, A., Itoh, T. and Itohara, S. (1995) *Neuron 14:*29-41.
21. van de Klundert, F.A.J.M., Raats, J.M.H. and Bloemendal, H. (1993) *Eur. J. Biochem. 214:*351-366.
22. Stewart, M. (1993) *Curr. Opinion Cell Biol. 5:*3-11.
23. Traub, P., Kühn, S. and Grüb, S. (1993) *J. Mol. Biol. 230:*837-856.
24. Heins, S. and Aebi, U. (1994) *Curr. Opinion Cell Biol. 6:*25-33.
25. Steinert, P.M., Steven, A.C. and Roop, D.R. (1985) *Cell 42:*411-419.
26. Raats, J.M.H. and Bloemendal, H. (1992) *Progr. Nucl. Acid Res. Mol. Biol. 43:*67-86.
27. Traub, P. and Vorgias, C.E. (1983) *J. Cell Sci. 63:*43-67.
28. Kaufmann, E., Weber, K. and Geisler, N. (1985) *J. Mol. Biol. 185:*733-742.
29. Perides, G., Kühn, S., Scherbarth, A. and Traub, P. (1987) *Eur. J. Cell Biol. 43:*450-458.
30. Andreoli, J.M. and Trevor, K.T. (1994) *Exp. Cell Res. 214:*177-188.
31. Rogers, K.R., Eckelt, A., Nimmrich, V., Janssen, K.-P., Schliwa, M., Herrmann, H. and Franke, W.W. (1995) *Eur. J. Cell Biol. 66:*136-150.
32. Hofmann, I. and Herrmann, H. (1992) *J. Cell. Sci. 101:*687-700.
33. Traub, P., Scherbarth, A., Wiegers, W. and Shoeman, R.L. (1992) *J. Cell. Sci. 101:*363-381.
34. Hatzfeld, M. and Weber, K. (1991) *J. Cell. Sci. 99:*351-362.
35. Letai, A., Coulombe, P.A. and Fuchs, E. (1992) *J. Cell. Biol. 116:*1181-1195.
36. Rothnagel, J.A., Dominey, A.M., Dempsey, L.D., Longley, M.A., Greenhalgh, D.A., Gagne, T.A., Huber, M., Frenk, E., Hohl, D. and Roop, D.R. (1992) *Science 257:*1128-1130.
37. Traub, P. and Shoeman, R.L. (1994) *Int. Rev. Cytol. 154:*1-103.
38. Kouklis, P.D., Papamarcaki, T., Merdes, A. and Georgatos, S.D. (1991) *J. Cell. Biol. 114:*773-786.
39. Georgatos, S.D. and Blobel, G. (1987) *J. Cell Biol. 105:*117-125.

40. Georgatos, S.D. and Blobel, G. (1987) *J. Cell. Biol. 105:*105–115.
41. Gotow, T., Tanaka, T., Nakamura, Y. and Takeda, M. (1994) *J. Cell Sci. 107:*1949–1957.
42. Nakagawa, T., Chen, J., Zhang, Z., Kanai, Y. and Hirokawa, N. (1995) *J. Cell Biol. 129:*411–429.
43. Muma, N.A. and Hoffman, P.N. (1993) *Micron 24:*677–683.
44. Geisler, N. and Weber, K. (1983) *EMBO J. 2:*2059–2063.
45. Traub, P., Mothes, E., Shoeman, R.L., Kühn, S. and Scherbarth, A. (1992) *J. Mol. Biol. 288:*41–57.
46. Traub, P., Mothes, E., Shoeman, R.L., Schröder, R. and Scherbarth, A. (1992) *J. Biomol. Struct. Dynamics 10:*505–531.
47. Traub, P. and Nelson, W.J. (1983) *Hoppe Seyler's Z. Physiol. Chem. 364:*575–592.
48. Traub, P., Nelson, W.J., Kühn, S. and Vorgias, C.E. (1983) *J. Biol. Chem. 258:*1456–1466.
49. Kumar, N.V. and Govil, G. (1984) *Biopolymers 23:*1995–2008.
50. Alberts, B., Frey, L. and Delius, H. (1972) *J. Mol. Biol. 68:*139–152.
51. de Jong, E.A.M., Duynhoven, J.P.M., Harmsen, B.J.M., Tesser, G.I., Konings, R.N.H. and Hilbers, C.W. (1989) *J. Mol. Biol. 206:*119–132.
52. Kühn, S., Vorgias, C.E. and Traub, P. (1987) *J. Cell Sci. 87:*543–554.
53. Paulson, J.R. and Laemmli, U.K. (1977) *Cell 12:*817–828.
54. Perides, G., Scherbarth, A., Kühn, S. and Traub, P. (1986) *Eur. J. Cell Biol. 41:*313–325.
55. Perides, G., Scherbarth, A. and Traub, P. (1986) *J. Cell Biol. 262:*268–280.
56. Perides, G., Harter, C. and Traub, P. (1987) *J. Biol. Chem. 262:*13742–13749.
57. Horkovics-Kovats, S. and Traub, P. (1990) *Biochemistry 29:*8652–8657.
58. Traub, P., Perides, G., Kühn, S. and Scherbarth, A. (1987) *Eur. J. Cell Biol. 43:*55–64.
59. Roise, D., Horvath, S.J., Tomich, J.M., Richards, J.H. and Schatz, G. (1986) *EMBO J. 5:*1327–1334.
60. Lithgow, T., Glick, B.S. and Schatz, G. (1995) *Trends Biochem. Sci. 20:*98–101.
61. Maduke, M. and Roise, D. (1993) *Science 260:*364–367.
62. Ouellet, T., Levac, P. and Royal, A. (1988) *Gene 70:*75–84.
63. Görlach, M., Meyer, H.E., Eisermann, B. and Soboll, S. (1995) *Biol. Chem. Hoppe-Seyler 376:*51–55.
64. Traub, P., Perides, G., Kühn, S. and Scherbarth, A. (1987) *Z. Naturforsch. 42c:*47–63.
65. Pruss, D., Hayes, J.J. and Wolffe, A.P. (1995) *BioEssays 17:*161–170.
66. Adams, C.C. and Workman, J.L. (1995) *Mol. Cell Biol. 15:*1405–1421.
67. Jones, N. (1990) *Cell 61:*9–11.
68. Lamb, P. and McKnight, S.L. (1991) *Trends Biochem. Sci. 16:*417–422.
69. Ellenberger, T.E., Brandl, C.J., Struhl, K. and Harrison, S.C. (1992) *Cell 71:*1223–1237.
70. Phillips, S.E.V. (1991) *Curr. Opinion Struct. Biol. 1:*89–98.
71. Starr, D.B. and Hawley, D.K. (1991) *Cell 67:*1231–1240.
72. Su, W., Jackson, S., Tjian, R. and Echols, H. (1991) *Genes Dev. 5:*820–826.
73. Mastrangelo, I.A., Courey, A.J., Wall, J.S., Jackson, S.P. and Hough, P.V. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88:*5670–5674.
74. Chen, J.D. and Pirrotta, V. (1993) *EMBO J. 12:*2075–2083.
75. Matthews, K.S. (1992) *Microbiol. Rev. 56:*123–136.
76. Chakerian, A.E. and Matthews, K.S. (1992) *Mol. Microbiol. 6:*963–968.
77. Chen, J.D., Chan, C.S. and Pirrotta, V. (1992) *Mol. Cell. Biol. 12:*598–608.
78. Benezra, R., Davis, R.L., Lockshon, D., Turner, D.L. and Weintraub, H. (1990) *Cell 61:*49–59.
79. Traub, P. and Shoeman, R.L. (1994) *BioEssays 16:*349–355.
80. Shoeman, R.L. and Traub, P. (1990) *J. Biol. Chem. 265:*9055–9061.
81. Christova, R., Bach, I. and Galcheva-Gargova, Z. (1992) *DNA Cell Biol. 11:*627–636.

82. Ludérus, M.E.E., deGraaf, A., Mattia, E., den Blaauwen, J.L., Grande, M.A., deJong, L. and van Driel, R. (1992) *Cell 70:*949-959.
83. Zeng, C., He, D. and Brinkley, B.R. (1994) *Cell Motil. Cytoskel. 29:*167-176.
84. Jackson, D.A. and Cook, P.R. (1988) *EMBO J. 7:*3667-3677.
85. He, D., Nickerson, J.A. and Penman, S. (1990) *J. Cell. Biol. 110:*569-580.
86. Wang, X. and Traub, P. (1991) *J. Cell Sci. 98:*107-122.
87. Willingale-Theune, J., Schweiger, M., Hirsch-Kauffmann, M., Meek, A.E., Paulin-Levasseur, M. and Traub, P. (1989) *J. Cell. Sci. 93:*651-665.
88. French, S.W., Kawahara, H., Katsuma, Y., Ohta, M. and Swierenga, S.H.H. (1989) *Electron Microsc. Rev. 2:*17-51.
89. Djabali, K., Portier, M.M., Gros, F., Blobel, G. and Georgatos, S.D. (1991) *Cell 64:*109-121.
90. Papamarcaki, T., Kouklis, P.D., Kreis, T. and Georgatos, S.D. (1991) *J. Biol. Chem. 266:*2147-2151.
91. Bader, B.L., Magin T.M., Freudenmann, M., Stumpp, S. and Franke, W.W. (1991) *J. Cell Biol. 115:*1293-1307.
92. Eckelt, A., Herrmann, H. and Franke, W.W. (1992) *Eur. J. Cell Biol. 58:*319-330.
93. Franke, W.W., Zentgraf, H., Scheer, U. and Kartenbeck, J. (1973) *Cytobiologie 7:*89-100.
94. Maison, C., Horstmann, H. and Georgatos, S.D. (1993) *J. Cell Biol. 123:*1491-1505.
95. Gooderham, K. and Jeppersen, P. (1983) *Exp. Cell Res. 144:*1-14.
96. Jones, J.C.R., Goldman, A.E., Yang, H.-Y. and Goldman, R.D. (1985) *J. Cell Biol. 100:*93-102.
97. Fidlerova, H., Sovova, V., Krekule, I., Viklicky, V. and Levan, G. (1992) *Hereditas 117:*265-273.
98. Bridger, J.M., Kill, I.R., O'Farrell, M. and Hutchison, C.J. (1993) *J. Cell Sci. 104:*297-306.
99. Wedrychowski, A., Schmidt, W.N., Ward, W.S. and Hnilica, L.S. (1986) *Biochemistry 25:*1-9.
100. Wedrychowski, A., Schmidt, W.N. and Hnilica, L.S. (1986) *J. Biol. Chem. 261:*3370-3376.
101. Cress, A.E. and Kurath, K.M. (1988) *J. Biol. Chem. 263:*19678-19683.
102. Hay, M. and DeBoni, U. (1991) *Cell Motil. Cytoskel. 18:*63-75.
103. DeBoni, U. (1988) *Anticancer Res. 8:*885-898.
104. Blessing, M., Rüther, U. and Franke, W.W. (1993) *J. Cell Biol. 120:*743-755.
105. Mizutani, A., Watanabe, N., Kitao, T., Tokumitsu, H. and Hidaka, H. (1995) *Arch. Biochem. Biophys. 318:*157-165.
106. van Holde, K. (1994) *Nature* (London) *367:*512-513.
107. Puck, T.T. and Krystosek, A. (1992) *Int. Rev. Cytol. 132:*75-108.
108. Chan, D., Goate, A. and Puck, T.T. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86:*2747-2751.
109. Abken, H., Hegger, R., Bützler, C. and Willecke, K. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90:*6518-6522.
110. Shoeman, R.L., Wadle, S., Scherbarth, A. and Traub, P. (1988) *J. Biol. Chem. 263:* 18744-18749.
111. Vissel, B. and Choo, K.H. (1989) *Genomics 5:*407-414.
112. Haran, T.E., Kahn, J.D. and Crothers, D.M. (1994) *J. Mol. Biol. 244:*135-143.
113. Wong, A.K.C. and Rattner, J.B. (1988) *Nucl. Acids. Res. 16:*11645-11661.
114. Stallings, R.L., Ford, A.F., Nelson, D., Torney, D.C., Hildebrand, E.E. and Moyzis, R.K. (1991) *Genomics 10:*807-815.
115. Manor, H., Rao, B.S. and Martin, R.G. (1988) *J. Mol. Evol. 27:*96-101.
116. Haaf, T. and Schmid, M. (1991) *Exp. Cell Res. 192:*325-332.
117. Spector, D.L. (1993) *Annu. Rev. Cell Biol. 9:*265-315.
118. Blackburn, E.H. and Szostak, J.W. (1984) *Annu. Rev. Biochem. 53:*163-194.
119. Vogt, P. (1990) *Hum. Genet. 84:*301-336.
120. Naylor, L.H. and Clark, E.M. (1990) *Nucl. Acids Res. 18:*1595-1601.

121. Kladde, M.P., d'Cunha, J. and Gorski, J. (1993) *J. Mol. Biol. 229:*344-367.
122. Albert, A.-C., Roman, A.-M., Bouche, G., Leng, M. and Rahmouni, A.R. (1994) *J. Biol. Chem. 269:*19238-19244.
123. Santra, M., Danielson, K.G. and Iozzo, R.V. (1994) *J. Biol. Chem. 269:*579-587.
124. Firulli, A.B., Maibenco, D.C. and Kinniburgh, A.J. (1994) *Arch. Biochem. Biophys. 310:*236-242.
125. Raghu, G., Tevosian, S., Anant, S., Subramanian, K.N., George, D.L. and Mirkin, S.M. (1994) *Nucl. Acids Res. 22:*3271-3279.
126. Hamada, H., Seidman, M., Howard, B.H. and Gorman, C.M. (1984) *Mol. Cell. Biol. 4:*2622-2630.
127. Opstelten, R.J.G., Clement, J.M.F. and Wanka, F. (1989) *Chromosoma 98:*422-427.
128. Wells, R.D., Collier, D.A., Hanvey, J.C., Shimizu, M. and Wohlrab, F. (1988) *FASEB J. 2:*2939-2949.
129. Herbert, A.G., Spitzner, J.R., Lowenhaupt, K. and Rich, A. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90:*3339-3342.
130. Pearson, C.E., Ruiz, M.T., Price, G.B. and Zannis-Hadjopoulos, M. (1994) *Biochemistry 33:*14185-14196.
131. Sen, D. and Gilbert, W. (1988) *Nature* (London) *334:*364-366.
132. Casasnovas, J.M., Huertas, D., Ortiz-Lombardia, M., Kypr, J. and Azorin, F. (1993) *J. Mol. Biol. 233:*671-681.
133. Pearlman, R.E., Tsao, N. and Moens, P.B. (1992) *Genetics 130:*865-872.
134. Wahls, W.P., Wallace, L.J. and Moore, P.D. (1990) *Cell 60:*95-103.
135. Knapp, S., Karth, G.D., Friedl, J., Purtscher, B., Mitterbauer, G., Domer, P.H. and Jaeger, U. (1994) *Oncogene 9:*1501-1505.
136. Allen, M.J., Jeffreys, A.J., Surani, M.A., Barton, S., Norris, M.L. and Collick, A. (1994) *Nucl. Acids Res. 22:*2976-2981.
137. Hutchison III, C.A., Hardies, S.C., Loeb, D.D, Shehee, W.R. and Edgell, M.H. (1989) In: "Mobile DNA" (D.E. Berg and M.M. Howe, ed), Am. Soc. Microbiol., Washington, DC, pp. 593-617.
138. Deininger, P.L. (1989) In: "Mobile DNA" (D.E. Berg and M.M. Howe, ed), Am. Soc. Microbiol., Washington, DC, pp. 619-636.
139. Wichman, H.A., Van Den Bussche, R.A., Hamilton, M.J. and Baker, R.J. (1992) *Genetica 86:*287-293.
140. Skowronski, J., Fanning, T.G. and Singer, M.F. (1988) *Mol. Cell. Biol. 8:*1385-1397.
141. Leibold, D.M., Swergold, G.W., Singer, M.F., Thayer, R.E., Dombroski, B.A. and Fanning, T.G. (1990) *Proc. Natl. Acad. Sci. US.A. 87:*6990-6994.
142. Gaubatz, J.W. (1990) *Mutation Res. 237:*271-292.

Physiological Chemistry and Physics and Medical NMR

INDEX

Volume 27, 1995

ABE, T., 131
ABKEN, H., 267, 321
ACALI, S., 251
β-N-acetylhexosaminidases,
  cell differentiation influence on, 369
  effect of 10-LEOYL-2-acetyl glycerol on, 262
  effect of phorbol ester TPA on, 262
  effect of protein kinase C activation on, 369
Acidic chromatin peptides,
  biological activity, 345
  molecular models, 345
  role in transcription, 345
Acidic (synthetic) peptides,
  phosphorylation by protein kinase CKII, 255, 363
  role in DNA transcription, 363
Acquired immunodeficiency,
  role of a lymphocytic low-molecular weight peptide in, 246
Actin motility,
  at nanomolar ATP levels, 167
ALBERTINI, G., 91
Aldehyde dehydrogenase,
  industrial solvent effect on activity of, 25
Alloxan,
  effect on lipid peroxide level, 149
  effect on SOD-activity, 149
Alloxan diabetes,
  protection against, by low-dose γ-irradiation, 149
ALONSO, A., 63
ALTAMURA, S., 243
ALTIERI, F., 313
AMALRIC, F., 268
AMICI, D., 250
Aminoethylcysteine ketimine dimer,
  as a free radical scavenger, 223
  as a lipid peroxidation inhibitor, 223
  properties of, 223
Amphibian skin secretions,
  antimicrobial peptides from, 244
Anesthetics, local
  effects on nitric oxide production, 111
Anesthetized hornets
  effect of light on sleep duration, 193
  extraretinal light perception by, 193
ANGIOLILLO, A., 255, 265, 266, 363
ANIASI, A., 246
Antibiotic peptides,
  identification, structure, biological activity of, 245
Antimicrobial peptides,
  from amphibian skin, 244
Antioxidative enzymes in liver,
  effect of industrial solvents on, 25
ANTONUCCI, A., 223
ATPase activity
  in DNA-binding protein complexes, 266
ATP, nonomolar levels,
  effect on actin motility, 167
ATP/GTP binding site,
  analysis of, by site-directed mutagenesis, 256, 293
AUBE, H., 240

β-turn mimics in peptide structure studies, 240
BAGELLA, L., 245
Balbiani Ring 2 genes,
  initiation of, 259
  productive transcription of, 259
BARBATO, G., 251
BARRA, D., 244
BARTOLONI, A., 253, 355
BAZZO, R., 238, 251
BIANCHI, E., 238, 243, 251

Bifonazole, effect on lipid matrix, 91
Blood pressure,
effect of low-dose γ-irradiation on, 161
BOLDYREFF, B., 254
BOSSI, G., 91
BOSSI, M.G.P., 91
BOUVET, P., 268
BRAMUCCI, M., 250
BRAY, A.M. 339
BUSCH, H., 258, 303

Calf liver mitochondria,
use in preparation of low molecular weight proteins, 248
Canavanine,
effect of nitric oxide generation, 111
Cancer cachexia,
use of NMR spectroscopy in monitoring, 226
CANOFENI, S., 244
CARDELLINI, E., 255, 303, 363
Cardiac myosins, actin motility in, 167
Casein kinase II, 254, 255, 256, 257, 293, 303, 363
ATP/GTP binding site of,
analysis by site-directed mutagenesis, 256, 293
molecular analysis of functional domains of, 254
phosphorylation sites in nucleolar protein p120, 303
See also Protein kinase CK II
CASTIGLI, E., 247, 345
Catalase,
industrial solvent effect on activity of, 25
CAVALLINI, D., 223
Cel biology, a commentary on, 139
Cell differentiation,
influence on β-N-acetylhexosaminidase distribution, 369
Cellular metabolic monitoring, perioperative
use of NMR spectroscopy in, 121
CENTINI, F., 252
Centrosomal proteins, a new family of, 265
CERVONI, L., 262, 313
CESARENI, G., 260
CHIMENTI, R., 248, 281
Chloroquinine,
binding constant in presence of surfactants, 1
interaction with ionic micells, 1
pK value of, 1
CIANFRIGLIA, M., 271
CICERO, D.O., 238
CILIBERTO, G., 243
CLEMENTI, M., 237
CLEMENTI, S., 237
Combinatorial libraries, 339
See also Peptide libraries
CORDERO, J.M., 217
CORTESE, R., 251
COVELLO, C., 248, 281
CRUCIANI, G., 237, 239

DAPREMONT, L.,238, 339
Database analysis,
PLANET as a tool for, 331
DEANGELIS, L., 247, 345
DE CICCO, T., 248, 281
DELMASTRO, P., 243
DE MAGISTRIS, M.T., 253, 355
DENTE, L., 260
Diabetes, alloxan, 149
5,6-dichloro-1-β-D-ribofuranosylbenzidazole (DRB),
as an agent in altering chromosomal distribution, 259
DNA,
calf liver mitochondrial,
use in preparation of LMW peptides, 248, 281
nuclear scaffold protein interaction with, 313
DNA-binding protein complexes,
high salt and SDS stable, 266
DNA sequences, short
in cytoplasm of Ehrlich ascites tumor cells, 267, 321
tight association with proteins, 267, 321
DNA transcription,
role of acidic peptides in, 345, 363
DRB,
as an agent in altering chromosomal distribution, 259
DRUCKER, Y.O., 73
DUBINI, B., 91
DURBAN, E., 258, 303
DUTTA, D., 240

EDASHIGE, K., 111
EGYHÁZI, E., 259
ELGJO, K., 249, 351

Electron spin resonance (ESR) spectroscopy,
as a technique in studies of stratum corneum and erythrocyte ghosts, 63
EMILIANI, C., 262, 369
Epidermal pentapeptide, 250, 351
characterization of, 351
Epitope mapping,
Multipin method of, 339
of MDR1-P-glycoprotein, 271
through use of phage-displayed peptide libraries, 271
use in detoxification of pertussis toxin, 355
ERARD, M., 268
EUFEMI, A., 264, 313
Erythrocytes, fluid shift in, 45
Erythrocyte deformability
and hyperbaric oxygen treatment, 45
Erythrocyte ghosts
comparison to stratum corneum intercellular lipids, 63

FELICI, F., 242, 251, 271
FERRARO, A., 264, 313
FIFI, P., 237, 239
FILIPPONI, E., 239
FINCHAM, C.I., 252
Flecainide acetate, effect on
action of hTNF-α, 77
human neutrophil priming, 77
L-929 cell growth, 77
FOLGORI, A., 242, 251
FORTUGNO, P., 242
Free radical scavenger,
ketamine dimer as a, 223
FUJIWARA, M., 131
FUKSMAN, E., 179
FUKUSHIMA, K., 121

Gene regulation,
role of intermediate filaments (IF) in, 263, 377
GENNARO, R., 245
GIANFRANCESCHI, G.L., 233, 247, 255, 345, 363
GIAVARINI, F., 247, 345
Glutathione peroxidase,
effect on activity by industrial solvents, 25
Glutathione reductase,
effect on activity by industrial solvents, 25
Glycoprotein, P-,
See P-glycoprotein
GOLDSTEIN, O., 193
GUSTAFSON, W.C., 258, 303
Gravity acceleration within plant tissue, 31
GREENLEAF, A.L., 259
Growth-modulating peptides, 249

HASHIMOTO, K., 203
HAZLEWOOD, C., 55
HEGGER, R., 267, 321
Hemorheology, 45
HENICS, T., 139
Hepatic metabolism changes,
cellular conditions on, 226
enzymatic abnormalities on, 225
Hexosaminidase
See β-N-acetylhexosaminidase
HINDS, T.R., 167
HIRAKI, Y., 149
HL-60 cells, β-hexosaminidases of, 262, 369
See β-N-acetylhexosaminidase
Hornet cuticle,
photoelectric current in, 179
thermoelectric current in, 179
Hornets,
extraretinal light perception by, 193
effect of light on sleep duration of, 193
HOSOI, Y., 17
Human neutrophil priming,
flecainide acetate effect on, 77
Hyperbaric oxygen treatment,
effect on red cell deformability, 45
effect on commonly measured blood parameters, 45
as cause of fluid shift in RBC, 45
Hypertension,
effect of low dose irradiation on, 161
Hypotaurine metabolism in rats, 131

IF proteins,
DNA interactions, 377
histone interactions, 377
lipid interactions, 377
role in gene regulation, 263, 377
See also Intermediate filaments
IMASATO, H., 1
Intercellular lipids of stratum corneum,
electron spin resonance studies of, 63
nitroxide reduction studies of, 63
thermotropic behavior studies of, 63

Intermediate filaments (IF),
binding of DNA fragments to, 377
biological role of, 377
cytoskeletal role of, 377
interaction with nucleus, 377
role in gene regulation, 263, 377
structure of, 377
See also IF proteins
ISHAY, J.S., 179, 193
ISHII, K., 17, 161
ISHIHARA, Y., 121
ISSINGER, O-G., 254

JAKOBI, R., 256, 293
JOSWIG, G., 265, 266
JUODKA, B., 266

KASTURI, S.R., 145
KELLERMAYER, JR., M.S.Z., 167
Ketimine
See Aminoethylcysteine ketamine
KHARRAT, A., 268
KITAGAWA, O., 240
KOBUCHI, H., 77
KODAMA, H., 203
KREAHN, A., 257
KRISTIANPOLLER, N., 193
KUMAR, S., 25
KURASHIGE, T., 203

L-929 cell growth,
action of flecainide acetate on, 77
Lanthidines,
effect on tubulin polymerization, 145
LEGUIZAMÓN, C.A., 217
LELONG, I.H., 261
LEPLAE, R., 331
Leukotriene B4,
low-dose X-irradiation effect on, 17
Lidocaine,
effect on nitric oxide generation, 111
Lipid membranes,
effect of bifonazole on, 91
Lipid peroxidation,
inhibition by ketimine dimer, 223
Lipid peroxide level,
effect of low-dose γ-irradiation on, 149
effect of alloxan on, 149
Liposome membranes,
bifonazole induced changes in, 91
LITINETSKY, L., 193
LIU, K.J.M., 73

Log P partition coefficient,
determination from 3-D-structure-water interactions, 239
LOMBARDI, P., 252
LORENZ, P., 257
Low-dose γ-irradiation, effect on
alloxan diabetes protection, 149
blood glucose level, 149
blood pressure of rats, 149
lipid peroxide level, 149
SOD activity, 149
Low-dose X-irradiation, effect on
Leukotriene B4 levels, 17
mitogen-proliferation response, 17
Low energy irradiated matter,
behavior following successive irradiations, 217
Low-molecular weight peptides,
from calf liver mitochondrial DNA, 248, 281
role in transcription process, 281
structure of, 281
LUGARO, G., 246, 247, 345
LUZZAGO, A., 242
Lymphocytic low-molecular weight peptide (LSF),
role in acquired immunodeficiency, 246

MACCARI, E., 250
MACHIDA, Y., 203
MAEJI, N.J., 339
MAESO, M.P., 237
MAMIYA, K., 111
MANABE, M., 111
MANCINELLI, L., 247, 345
MARSILI, V., 247, 345
MARTINDALE, V.E., 45
MARTINO, G., 248, 281
MARTINO, S., 248, 281
MASON, T.J., 238, 339
MASUOKA, N., 203
MAZARGUIL, H., 268
MAZZULLA, S., 248, 281
McKAY, K., 45
MDR1-P-glycoprotein,
in monoclonal antibody structure studies, 271
mapping of epitope of, 271
MEGGIO, F., 254
MEIRELLES, N.C., 63
MENNUNI, C., 242

Methyl alcohol, effect on antioxidative enzymes in liver, 25
MIANO, A., 250
MIGNOGNA, G., 244
Mitochondrial DNA, 248, 281, 313
low molecular weight protein from, 248, 281
Mitogen-induced proliferation,
of rat splenocytes, 17
enhancement by low-dose X-irradiation, 17
Model membranes,
effect of biofonazole on, 91
Molecular interactions
by phage-displayed peptides, 242
Molecular models of peptides,
by mass spectrometry analysis, 247
Monoclonal antibody epitope MM4.17,
structural conformation of, 271
MONTE, S.C., 1
MONTEAGUDO, E., 252
MONTECUCCHI, P.C., 245
MONTEFOSCHI, G., 223
MOON, L.J., 259
MORTON, M., 240
Multidrug resistance,
transporter responsible for, 261
p-glycoprotein as transporter responsible for, 261
Multipin system in peptide multiple synthesis, 339
Multiple synthesis of peptides,
use of multipin method in, 339
in future drug discovery, 339
MURRI, O., 250

Neutrophils,
effect of local anesthetics on, 111
NICOTRA, M., 251
Nitric oxide production,
effect of local anesthetics on, 111
Nitroxide reduction in stratum corneum, 63
NK-1 substance P receptor antagonist,
molecular model of, 252
NMR spectroscopy, use in
bifonazole action studies, 91
cancer cachexia, 226
monitoring cellular environment, 73
peptide structure determination, 238
perioperative cellular metabolic monitoring, 121
serum metabolite studies, 121
NOGUCHI, J., 121
NOJIMA, S., 77
Non-peptide polymer library, 339
Nuclear proteins, a new family of, 265
Nuclear scaffold attachment regions (SAR) of DNA,
protein/DNA interactions at, 264, 313
UV irradiation studies of, 313
Nuclear scaffold proteins,
interaction with DNA, 264, 313
UV irradiation studies of, 313
Nucleolar phosphoprotein p 120,
characterization of, 258, 303
functional domains of, 303

OHKOCHI, J., 121
Oligopeptides, pyroglutamyl N terminal,
characterization of, 351
ONO, T., 17
ORLACCHIO, A., 262, 369
OSSOINAK, A., 259

PALUMBO, S., 271
PAOLINI, C., 242
PECCI, L., 223
PEPPOLONI, S., 253, 355
Peptide libraries, 238, 241, 242, 243, 251, 260, 331, 339
a conformationally homogeneous combinatorial, 251
evaluation of, 238
M-SPCL (multimeric synthetic peptide combinatorial), 243
use in protein structure studies, 241, 242
use in tyrosine phosphorylation specificity studies, 255
See also Phage peptide libraries
Peptide mimics,
in the study of antigen-binding, 242
Peptide molecular models, 247
Peptides, 251, 255, 281, 345
acidic chromatin peptides, 345
low-molecular weight from mitochondrial DNA,
role in transcription, 281
structure of, 281
phosphorylation by protein kinase CKII, 255
selection from combinatorial peptide library, 251
Peptide secondary structure,
role of β-turns, 240

Peptide selection
from combinatorial peptide library, 251
Peptide sequences
produced by phage libraries, 331
Periodic Continuous Effect, 217
Pertussis toxin,
detoxification of, 355
genetically inactive, 253, 355
use in pertussis vaccine, 253, 355
Pertussis vaccine, acellular,
genetically modified, 253, 355
PERUSSI, J.R., 1
PESSI, A., 238, 243, 251
P-glycoprotein,
as transporter responsible for multidrug resistance, 261
ATP/GTP-phosphorylation of, 261
PHADKE, R.S., 91
Phage peptide libraries,
in mapping MDRI- P-glycoprotein epitope, 271
in tyrosine/phosphorylation specificity studies, 260
PLANET as analysis tool for, 331
software system for database analysis of, 331
See also Peptide libraries
PHALIPON, A., 251
Phase transition
in stratum corneum lipid moiety, 63
Phosphorylation of peptides
by protein kinase CKII, 363
Photoelectric current in hornet cuticle, 179
PIGON, A., 259
PINNA, L.A., 254
PIZZA, M., 253, 355
PIZZI, E., 241
PLANET
a phage library analysis tool, 331
software system for database analysis, 331
POLLACK, G.H., 167
POLONI, F., 271
Poly-L-proline,
scanning tunneling microscopy study of, 55
Polymerization, tubulin,
effect of lanthanides on, 145
POTIER, E., 252
Primaquine,
binding constant in the presence of surfactants, 1
interaction with ionic micells, 1
interaction with surfactants,1
pK value of, 1
Pyroglutamyl N-terminal oligopeptides
as part of larger molecules, 249
D,L-proparaglyglycine treated rats,
cystathionine metabolites in urine of, 203
Protein-DNA interactions,
at nuclear scaffold attachment regions of DNA, 313
UV irradiation studies of, 313
Protein kinase activity
in DNA-binding protein complexes, 266
Protein kinase C,
effect on β-N-acetylhexosaminidase distribution, 369
phosphorylation sites in nuclear protein p120, 303
Protein kinase CKII (CK2), 254, 255, 256, 257, 293, 303, 363,
crosslinking of subunit contact sites in, 257
function of, 257
molecular analysis of functional domains of, 254
peptide-based immunomapping in, 257
phosphorylation of acidic peptides, 255, 363
See also Casein kinase II
Protein p120,
See Nucleolar phosphoprotein p120
Protein stability in culture media, 246
PUGNALONI, A., 91
PYERIN, W., 257

QSAR studies of proteins, 237
QUASSINTI, L., 250

RAGANELLI, D., 237
RAI, S.S., 145
RANA, S.V.S., 25
RAPPUOLI, R., 253, 355
RAU, C., 55
RAU, G., 55
REBEL, G., 246
REICHELT, K.L., 249, 351
REICHELT, W.H., 249, 351
REN Y., 258
RISSO, A., 245
RNA polymerase II,
C-terminal domain phosphorylation, 259

RNA, single stranded,
interaction with RNP-1 consensus sequence, 268
structural determinants of, 268
RNP-1 consensus seqeunce,
interaction with single-stranded RNA, 268
structural determinants of, 268
ROMEO, D., 240
ROSENZWEIG, E., 179
ROSSO DI SAN SECONDO, V.E.M., 246
ROTHBARTH, K., 266

SAIBARA, T., 77
SAKAMOTO, K., 17
SANTINI, C., 242
Sap flow in trees, theory of, 31
SAR binding proteins, 313
SARNO, S., 254
SAR-nuclear matrix interactions,
UV studies of, 313
Scanning tunneling microscopy (STM)
of poly-L-proline, 55
SCOCCHI, M., 245
Serum metabolites,
perioperative concentration changes in, 121
proton NMR spectroscopy studies of, 121
SGARAMELLA, G., 242
SIMMACO, M., 244
SIRCHIA, G., 245
SISTO, A., 252
Site-directed mutagenesis,
analysis of binding site of casein kinase II, 293
Skeletal myosins, actin motility in, 167
SKERLAVAJ, B., 245
SOD-activity,
effect of low-dose irradiation on, 149
effect of alloxan on, 149
SPENA, A., 248, 281
SPIESS, E., 266
SRIVASTAVA, S., 91
STEWART, K.M., 339
STIRLING, J.L., 262, 369
STORICI, P., 245
Stratum corneum,
comparison to erythrocyte ghosts, 53
electron spin resonance (ESR) studies of, 63
nitroxide reduction in, 63
thermotropic behavior studies of, 63
Substance P receptor antagonists,
molecular model of, 252
Superoxide dismutase activity
See SOD
Superoxide generation
of human peripheral neturophils, 77
Swine serum enzyme,
purification and characterization of, 250
hydrolyses of epidermal inhibitory pentapeptide, 250

TABAK, M., 1, 63
TAKAHARA, Y., 149
TAKUSAGAWA, F., 240
TATARA, T., 121
Taurine formation from hypotaurine
oxidation, 131
TAYLOR, C.W., 303
TERRACIANO, R., 252
Thermoelectric current in hornet cuticle, 179
Three-D (3D)-QSAR studies of proteins, 237
Three-D (3-D)-structure-water interactions, 239
log P determination from, 239
Toluene, effect on antioxidative enzymes
in liver, 25
TOMODA, M.K., 111
TOMOZAWA, M., 131
TONIATTI, C., 243
TOSSI, A., 245
TRAMONTANO, A., 241, 331
Transcription,
role of acidic chromatin peptides in, 345
See also DNA transcription
TRAUB, P., 263, 377
TRAUGH, J.A., 256, 293
Tubulin polymerization,
effect of lanthanides on, 145
Tumor necrosis factor-α (hTNF-α)
flecainide acetate inhibition of action of, 77
TURANO, C., 264, 313

UBUKA, T., 131, 203
UEDA, W., 111
UEDAIRA, H., 121
Urine, cystathionine metabolites in, 203
UTSUMI, K., 77, 149

VAGLIO, P., 254
VALDEZ, B.C., 258, 303
VALERIO, R.M., 339
VALIGI, R., 240

VANDER VELDE, D., 240
Vespa orientalis,
effect of light on sleep duration, 193
extraretinal light perception by, 193
photoelectric current in cuticle of, 179
thermoelectric current in cuticle of, 179
VETRIANI, C., 260

WAGNER, O.E., 31
WAKIGUCHI, H., 203
WALLACE, A., 238, 243, 251
WANG, A.W., 339
WERNER, D., 265, 266
X-irradiation, low-dose,
See Low-dose X-irradiation
X-ray diffraction as a technique in studying bifonazole action, 91
Xylene, effect on antioxidant enzymes in liver, 25

YAMAOKA, K., 17, 149, 161
YASUDA, T., 77
YOSHIOKA, T., 149
YUKIHIRO, K., 131
YUSHMANOV, V.E., 1

ZANETTI, M., 245
ZHANG, J., 203
ZHENG, N., 55

Volume 27, Number 4, 1995

Continued from back cover

MINIREVIEWS

R. LEPLAE and A. TRAMONTANO PLANET: A Phage Library Analysis Expert Tool 331

L. DAPREMONT, R.M. VALERIO, A.M. BRAY, K.M. STEWART, T.J. MASON, A.W. WANG and N.J. MAEJI Multiple Synthesis Using the Multipin Method 339

G. LUGARO, L. DE ANGELIS, F. GIAVARINI, V. MARSILI, L. MANCINELLI, E. CASTIGLI and G.L. GIANFRANCESCHI Acidic Chromatin Peptides. Molecular Models and Biological Activity 345

K. ELGJO, K.L. REICHELT and W.H. REICHELT Biologically Active Pyroglutamyl N-Terminal Oligopeptides: Parts of Larger Molecules? 351

S. PEPPOLONI, M. PIZZA, M.T. DE MAGISTRIS, A. BARTOLONI and R. RAPPUOLI Acellular Pertussis Vaccine Composed of Genetically Inactivated Pertussis Toxin 355

E. CARDELLINI, A. ANGIOLILLO and G.L. GIANFRANCESCHI Phosphorylation of Acidic Peptides by Protein Kinase CKII 363

C. EMILIANI, S. MARTINO, J.L. STIRLING and A. ORLACCHIO Influence of Cell Differentiation and Protein Kinase C Activation on Sub-Cellular Distribution of β-N-Acetylhexosaminidases of HL 60 Cells 369

P. TRAUB Intermediate Filaments and Gene Regulation 377

INDEX, Volume 27 (1995) 401

Physiological Chemistry and Physics and Medical NMR

Volume 27, Number 4, 1995

CONTENTS

Proceedings of the 1st International Symposium Trends in Peptide Research

Introduction
Gian Luigi Gianfranceschi 233

ABSTRACTS 235

ARTICLES

F. POLONI, S. PALUMBO, M. CIANFRIGLIA and F. FELICI Selection of Phage-Displayed Peptides Mimicking an Extracellular Epitope of Human MDR1-P-Glycoprotein 271

A. SPENA, R. CHIMENTI, C. COVELLO, T. DE CICCO, S. MAZZULLA and G. MARTINO Low Molecular Weight Peptide from Calf's Liver Mitochondrial DNA: Structure and Effect on DNA as a Template 281

R. JAKOBI and J.A. TRAUGH Analysis of the ATP/GTP Binding Site of Casein Kinase II by Site-directed Mutagenesis 293

E. DURBAN, B.C. VALDEZ, W.C. GUSTAFSON, C.W. TAYLOR, E. CARDELLINI and H. BUSCH Functional Domains of Nucleolar Phosphoprotein p120 303

A. FERRARO, M. EUFEMI, L. CERVONI, F. ALTIERI and C. TURANO Protein-DNA Interactions at the Nuclear Scaffold Attachment Regions of DNA Loops 313

R. HEGGER and H. ABKEN The Short DNA Sequences in the Cytoplasm of Ehrlich Ascites Tumor Cells Are Tightly Associated with Proteins 321

Continued inside the back cover

Printed in the U.S.A.