Division Pathochemistry (B0100 / A060)

Head: Prof. Dr. Volker Kinzel

Senior Scientists
Dr. Dirk Bossemeyer
PD Dr. Dieter Kübler
Prof. Dr. Jennifer Reed

Post-docs
Dr. Michael Gaßel (5/01 -)
Dr. Dan Mihailescu (- 12/01)

Guest Scientists
Dr. Saturnino Herrero, Spain (1/01 -)
Dr. Dan Mihailescu, Romania (1/02 -)

Graduate and Postgraduate Students
Susanne Elsner (4 - 9/02)
Katja Gehenn (2 - 9/02)
Darko Goswenca (- 8/02)
Marion Mohl (2 - 6/02)
Andreas Schlosser (together with R0400)

Technicians
Hannelore Horn (½)
Norbert König
James Richards

The Division focuses on specific molecular aspects of cellular signal transduction aimed at the identification of therapeutic targets. The projects include the analysis of the structural control of molecular interactions, of enzyme regulated interactions of the cell surface with the extracellular compartment, and last but not least the elaboration of the molecular basis for the therapeutic inhibition by drugs of protein kinases central in malignant growth.

Cell surface protein kinases and extracellular phosphorylation

D. Kübler, V. Kinzel

in cooperation with Prof. Dr. W.-D. Lehmann, M. Wind, Dr. H. Heid, DKFZ; Prof. Dr. I. Friedberg, University of Tel Aviv, Israel

Cell growth, differentiation and cell death are critically regulated by extracellular signalling processes which include the activity of various cell surface enzymes. Over the last years we have established the existence of externally oriented protein kinase (PK) activities having properties of cAMP-dependent PK (PKA) and cyclic nucleotide-independent PK (CK1 and CK2). Ecto-CK1/CK2 but not ecto-PKA can be released in tandem from intact cells into the extracellular compartment. Through these ecto-PK activities, cells are enabled to modulate cell surface proteins as well as substrates from the extracellular environment and thereby specifically alter biological functions. A steadily increasing amount of literature data on normal and pathological processes support the central role of extracellular protein phosphorylation. These findings suggest that not only is phosphorylation used as a mechanism of extra-intracellular signalling but that ecto-PKs and their substrates might serve as important pharmacological and therapeutic targets.

Ecto-PK substrates at the cell surface can be radioactively labeled and, after gel electrophoresis and autoradiography, show up as cell specific patterns of phosphoproteins. Their identification and understanding of their biological function(s) is of prime importance. Recent results of biochemical and immunological analysis including microsequencing and techniques of mass spectrometry showed a further highly phosphorylated cell surface protein to be a homolog of a known nucleolar protein, thus indicating multicompartmental functions[1]. The characterization of this and other ecto-substrates, their expression as cell surface proteins and comparative investigations with normal and tumor cells are important topics of our ongoing research.

Among the extracellular ecto-PK substrates are molecules with functions as different as growth factors, hormones, inflammatory proteins or components of the extracellular matrix. The collaborative research with I. Friedberg (Tel Aviv) on the mechanisms of extracellular nucleotides (ATP) as inducers of cell growth arrest has linked a putative inhibitory protein with the inactivation of growth factor-coupled tyrosine kinase activity. The phosphorylation by ecto-PK of the latent inhibitor in the extracellular fluid, which has been identified as a plasma glycoprotein derivative, appears to be central for the cell proliferation inhibition process. The definition of mechanisms which lead to the biological production of this growth inhibitor from precursors and its phospho-activation as well as downstream signalling are further steps for understanding, and probably influencing, the ectophosphorylation-mediated cell proliferation inhibition.
Bioregulation of the cAMP-depending protein kinase

M. Gaßel, S. Herrera de Vega, N. König, V. Kinzel, D. Bossemeyer

In collaboration with Dr. R. A. Engh, Roche-Diagnostics Penzberg; Prof. Dr. R. Huber, Dr. T.A. Holak, MPI für Biochemie, Martinsried; and Prof. Dr. W.-D. Lehmann, DKFZ

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Many human diseases, including the majority of all cancers, are connected to the deregulation of certain protein kinases. Protein kinases, as key elements of cellular regulation and signal transduction, phosphorylate other proteins in order to reversibly change their cellular function. The basic state of most protein kinases, however, is the state of inactivity - pathological effects result from overexpression or overactivation or loss of inhibitory control. Protein kinase inhibitors, therefore, are highly promising and, in some cases, already very successful agents in tumor therapy. This is demonstrated by the example of the protein kinase inhibitor gleevec (STI571), used for the treatment of chronic myeloid leukemia, which is extremely efficient with very few side effects. Structure based approaches are increasingly important for the development of such therapeutics. The bottleneck in the development of selective protein kinase inhibitors is the availability of crystal structures from therapeutically relevant protein kinases and structural information about their interaction with protein kinase inhibitors. From the 518 different kinases of the human genome, only little more than 5% have been crystallized so far. The research group therefore follows several strategic lines to achieve a deeper understanding of the biological key functions of some of these enzymes.

The high degree of conservation of the protein kinase catalytic core allows us to simulate certain properties and structural characteristics of other, related kinases by site directed mutagenesis of PKA (leading to so called surrogate kinases) [Selectivity determinants of the ATP binding site of PKB induce a critical re-arrangement when introduced into PKA, Gaßel et al., submitted]. For a solid and broad basis for the design and development of new inhibitors we try to achieve a deeper understanding of the structural and functional mechanism of catalysis and the cellular processes of kinase inactivation. For this purpose we combine structural investigations [3], site directed mutagenesis, and functional as well as kinetic studies (surface plasmon resonance). Regulation of protein kinases is achieved in many cases by phosphorylation of the enzyme itself. PKA has, depending on the isoforms, four or five phosphorylation sites. We analyzed these sites by various means [4,5,6], including NMR-spectroscopy [7].

The biological effect of many hormones is mediated by changes in the intracellular level of cAMP. The primary target of cAMP is one enzyme, PKA. The numerous and specific cellular responses to each hormone via the second messenger cAMP can only be explained by assuming different subcellular distributions and individual properties of PKA and its isoforms. Genetically encoded are Cα, Cβ, Cγ, and Cβ2; the latter was discovered and cloned in the department. There are also other splice variants. Cβ2 is unique, because it contains an aminoterminal extra domain (60 amino acids). At present we investigate the role of Cβ, and Cβ2 with respect to their biochemical properties and biological function of regulation, protein protein interaction, translocation and substrate recognition. We found that the Cβ2 protein is ubiquitous in mammals and present even in birds and can compensate some functions of Cα in Cα negative cells (Thullner et al., Biochemical Journal 351 (2000) 123-32).

On the protein level, two isoelectric charge variants of PKA were detected and characterized. This led to the discovery of a conserved in vivo deamidation site at the amino terminus of all myristoylated PKA isoforms, using mass spectrometry techniques, as published previously. Our understanding of the functional role of this deamidation site becomes clear from an investigation on living cells: it determines the intracellular distribution and translocation of the PKA C-subunit. Consequently, the phosphorylation of the transcription factor CREB (cAMP response element binding protein) becomes changed (Pepperkok et al., J. Cell Biol. 148 (2000) 715-26). In addition, the immediate process of deamidation, which includes racemization steps, was analyzed in close detail (Lehmann et al., Protein Science 9 (2000) 2260-68; Kinzel et al., Protein Science 9 (2000) 2269-77); [8].

Taken together, the two central scopes of the research group are: 1. to provide a thorough structural basis for the development of new and selective protein kinase inhibitors for the treatment of cancer and other diseases. 2. to achieve a deeper understanding of the biological key function of PKA and its isoforms in the important and complex cAMP-signaling pathway.

Regulation of protein phosphatase 2A (PP2A) at the G2-mitosis transition

V. Kinzel, N. König, J. Richards

In collaboration with Prof. Dr. W. D. Lehmann, A. Schlosser, M. Wind, M. Saleik, DKFZ

Multicellular organisms control the replication of their cells through a mostly reversible inhibition and stimulation at the physiological restriction points within the cell cycle - in G0 and partly in G2 prior to mitosis (M). Since protein phosphorylation and dephosphorylation events play a key role in the respective regulation, the control of the cytokines, protein kinases and phosphatases, at the molecular level is of major importance.
We are studying the regulation of PP2A at the G2-mitosis transition. Preliminary evidence points to changes in enzyme activity and certain posttranslational modifications of the PP2A catalytic subunit as the cell enters division. The aim is to characterize in detail the enzyme isolated from cells in different cell cycle phases by mass spectrometry.

Structure determinants of proteins

J. Reed, K. Weise, K. Domgall, D. Mihailescu

In cooperation with Prof. Dr. J.C. Smith, IWR, Heidelberg; Prof. Dr. D. Langosch, Technische Universität, Munich; Prof. Dr. H. de Groot, Leyden University; Prof. Dr. K. Gerwert, Universität Bochum; Prof. Dr. G. Multhaup, Freie Universität Berlin

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The rich source of information provided by the complete solution of the human genome and a growing number of animal genomes cannot properly be tapped without knowledge of the gene products: what they are and what functions they carry out. In principle, all information needed to determine the three-dimensional structure of a protein is contained within its amino acid sequence, i.e., the trinucleotide codons of the gene. From the sequence and the structure one can then often determine the function of the gene product. In practice, we do not yet - even after more than fifty years - have all the knowledge necessary to predict protein structure from sequence. Yet this is of vital importance, because the rate of structure resolution using NMR and X-ray crystallography is far too slow to give us the information we need within a reasonable amount of time. Every bit of information we can add to our knowledge of protein folding and protein structural motifs is urgently needed.

A particularly important level of protein structure is the domain, a level lying between simple secondary structure elements and the fully folded tertiary structure. Much smaller than the parent protein, domains are often independent folding units, tend to correspond to exons, and retain in isolation from the rest of the protein - their particular functions. For this reason they are excellent study subjects for understanding both protein folding and the relationship between structure and function. Several types of domain or motif are presently under study in this group.

The conformational switch in gp 120 from HIV1

This laboratory discovered and has extensively investigated a conformational switch within the primary CD4-binding domain of the HIV1 envelope glycoprotein gp120 (Reed and Kinzel Biochemistry 30, (1991) 4521-4528). The switch is a 15 residue sequence with the conserved ability to refold reversibly and in a cooperative manner from β-sheet in polar (serum-like) conditions to helix when in an apolar (membrane-like) environment. The switch domain can on its own bind specifically to CD4-expressing cells, but any mutation or externally applied substance that undermines the cooperativity of the folding transition - the switch - also prevents binding. This finding has been used to develop and optimise a series of “switch inhibitors” that prevent binding of the gp120 fragments to CD4-expressing cells (Reed and Kinzel Biochemistry 33, (1994) 10993-10998; von Stosch et al., Biochemistry 35 (1996) 411-417). These inhibitors are being patented and further development is being carried out in cooperation with the Steinbeis GmbH (Transfer Center for Applied Biological Chemistry and for Glycoconjugates).

In the mean time, the fact that the secondary structure under serum-like conditions (β-sheet) was conserved despite variations in amino acid sequence among HIV1 clades suggested a novel strategy for vaccine development. If the full structure of the switch sequence from one strain could be solved by NMR or crystallography, the sequences of other strains representing the full spectrum of clades could be threaded on to this backbone and the resulting surfaces compared for conserved features (local charge, hydrophobic patches, etc.) that could be used to design a strain-independent antigen. A problem with this strategy is that the very high tendency of the polar form of the switch to aggregate renders both classic structural approaches impossible. We have solved this problem by determining the structure of the switch peptide in organic solvents using NMR [9]. In three different organic solvents of different polarities (DMSO, TFE and Methanol:CCl₄) we obtained three different structures of the switch peptide from strain LAV. We then used molecular modelling techniques to place each of these in an explicit water box and observed the refolding process until equilibrium was reached. If all three, starting from different structures, arrived at the same or closely similar structure when placed in virtual water, we could conclude with a high level of confidence that this structure was in fact the one naturally adopted in water.

This approach was successful, the three structures converging to a single structure with an rms deviation of less than 2 Å over the majority of the backbone [10]. This structure has been used to thread 12 sequences from 9 clades of HIV1 and the surfaces compared. A constant pharmacophoric footprint does exist, and we are in the process of defining this precisely for use in the design of antigens for vaccine development.

Preliminary results have shown that polarity driven conformational switches of the type described above are not unique to gp120 and examples have been found in a number of other proteins (Lindemann et al., Proteins: Structure, Function and Genetics 29 (1997) 203-211; Domgall et al., submitted). Some of these are of particular interest from the medical point of view; for instance, we have shown a conformational switch exists in the transactivating domain of the tumor suppressor protein p53. Accordingly, we intend to extend our research in this direction to determine how common conformational switches are, what types exist and in what proteins they occur. This information will be useful not only in identifying potential useful drug targets, but also in adding to the lists of known protein motifs used in protein structure prediction.
Post-translational modifications

One aspect ignored by all present approaches to structure prediction from sequence is the effect of post-translational modifications on protein structure and function. Using circular dichroism and NMR, we have carried out measurements on synthetic peptides to examine systematically the effect of phosphorylation in particular but also of other naturally-occurring modifications such as myristoylation and deamidation. We have been able to show for the first time that phosphorylation directly alters the preferred backbone dihedral angle $\phi$ of the affected residue (Tholey et al., *Biophys. J.* (1998) **76**, 76-87). Experiments mimicking the multiple modifications within the N-terminal segment of cAMP-dependent protein kinase have shown how they can affect structure and membrane affinity in a synergistic manner [6].

Fusogenic peptides

A type of domain of especial interest due to its involvement both in normal cellular (synaptic vesicles, etc.) and pathogenic (viral entry) processes is that which drives the fusion of membrane bilayers. The sequences that promote fusion are fairly short, in the order of 16-20 residues, and the mechanism by which they induce bilayer fusion is not fully understood. In a project funded by the VW Stiftung and in collaboration with laboratories in Munich, Bochum and Leyden we are examining natural and designed synthetic fusion peptides and examining them for the connection between fusogenic activity and conformational properties using CD, FTIR and NMR. Initial studies on natural SNARE transmembrane segments showed a clear connection between fusogenicity and an association driven by the principal CD4 binding domain of gp120. J. Med. Chem. 45, 1019-1025

External collaborations

The usefulness of circular dichroism in determining secondary structure content and conformational changes in proteins under near physiological conditions while requiring relatively low sample concentrations means that the expertise within this group is in demand for a number of external collaborations. Some of the most recent include studies on the E5 protein in human papilloma virus, protease specificity in spuma viruses, the connection between conformation and pathogenicity in the amyloid $\beta$ peptide in Alzheimer’s disease and chaperone interactions (Schneikert et al, *EMBO J.* (2000) **19**, 6508-6516) [11,12,13].