Regulating the regulators

Conference on protein phosphorylation and protein phosphatases

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The EMBO Conference on 'Protein Phosphorylation and Protein Phosphatases' was held in Marburg, Germany, July 8–12, 2001. The organizers were Susanne Klumpp (University of Marburg), Joaquin Arino (University of Barcelona) and Mathieu Bollen (University of Leuven). Next year the meeting will go to the USA. It will be organized by Ben Neel (bneel@caregroup.harvard.edu) and Angus Nairn and will be held at Snowmass, CO, July 13–18, 2002. The 2003 conference will be held in Europe again, at Sitges, near Barcelona, Spain, under the management of Joaquin Arino (joaquin.arino@uab.es), Susanne Klumpp and Denis Alexander.

PPP and PPM subfamily members utilize the same binuclear metal assisted catalytic mechanism, they appear to have emerged through convergent evolution. Tyr-specific protein phosphatases (PTPs) have been identified since and are composed of transmembrane (receptor-like) and cytosolic (non-receptor) subfamilies. These phosphatases not only counter-balance the effects of receptor and cytosolic protein Tyr kinases but also transmit information through the plasma membrane. Thus, they are of paramount importance in cellular signaling. Like the broad-substrate-specificity or dual-specificity protein phosphatases (DSPs), PTPs contain a highly conserved cysteine residue that is essential for the catalytic reaction. In contrast to the relatively well studied processes of Ser/Thr and Tyr phosphorylation, histidine (His) phosphorylation in eukaryotes is an emerging field, and a unique protein phosphatase responsible for His dephosphorylation (PHP) has been identified recently.

In order to counteract the large number of protein kinases, protein phosphatases exhibit broad substrate specificity and interact with numerous regulatory and targeting proteins that control the activities of phosphatases by changing the conformation and location of these enzymes. Since the discovery of protein phosphorylation–dephosphorylation as a possible regulatory device in the mid-1950s, there has been a steady expansion of the field. The latest developments were summarized and new research directions were identified at this EMBO Conference on 'Protein Phosphorylation and Protein Phosphatases' held in Marburg, Germany.

Introduction

According to a modest estimation, one third of the known eukaryotic proteins can be phosphorylated. Protein kinases are members of a large superfamily and incorporate the phosphate moiety into the serine (Ser), threonine (Thr) or tyrosine (Tyr) residues of their target proteins. In comparison, protein phosphatases are less numerous but more diverse. They can be classified into four independent protein families according to their substrate specificities, catalytic mechanisms and amino acid sequences (Figure 1). The phosphatases specific for Ser and Thr side chains were first identified in classical biochemical studies, but their subsequent cloning revealed significant structural differences between the family members, which led to their division into the phosphoprotein phosphatase (PPP) and metal-ion-dependent protein phosphatase (PPM) subfamilies. Molecular cloning and genetic studies also led to the discovery of the so-called novel PPP enzymes. Despite the fact that both

Regulatory protein complexes

It is probable that with the flurry of interest in genomics and proteomics, all of the protein kinase and phosphatase catalytic subunits or domains will soon be ascertained. Obviously, subsequent experiments will need to address their roles under

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Fig. 1. Protein phosphatase families. PPP, phosphoprotein phosphatase; PPM, metal-ion-dependent protein phosphatases; PTP, Tyr protein phosphatases; DSP, dual-specificity phosphatases; PHP, His protein phosphatases. The distribution of the first identified classical PPP members (PP1, PP2A, PP2B and PP2C) is indicated. Horizontal bars represent substrate specificity.

physiological conditions and to identify their interacting partners.

In his opening lecture, Tony Pawson (Toronto, Canada) presented several interesting examples of phosphorylation-dependent protein-protein interactions. A wide variety of Src homology 2 (SH2)-domain-containing proteins (enzymes, onco-proteins, scaffolds, adaptors, regulators and transcription factors) bind phosphorylated Tyr residues, whereas others (e.g. 14-3-3 proteins and forkhead-associated domains, as well as WW and WD motifs) are specific for phospho-Thr and phospho-Ser. As a result, the phosphorylation-dephosphorylation state of a protein can profoundly affect its localization and redistribution. An increasing number of such interacting proteins or domains have been identified and their roles in signal transduction, cytoskeletal reorganization, synaptic regulation, protein trafficking and cell cycle progression were discussed. Particularly interesting in this respect was Pawson's demonstration of the significance of such protein-protein interactions in the rearrangement of the actin cytoskeleton following pathogenic *Escherichia coli* infection. One take-home message distilled from his experiments was that several weaker interaction sites are more effective regulators than is a single strong binding site; the former arrangement is more flexible and versatile and provides opportunities for cooperative binding. In support of the importance of these interactions in general, David Barford (London, UK) demonstrated the phosphorylation-dependent complex formation between the kinase-associated dual-specificity phosphatase (KAP) and the cyclin-dependent kinase 2. His X-ray crystallographic pictures highlighted the significance of the phosphorylated Thr residue of the kinase activation loop as the most important area of interaction.

Compartmentalization of protein kinases and phosphatases is a key factor in signaling (Figure 2). The A-kinase anchoring proteins (AKAPs) function as mediators between the conflicting activities of kinases and phosphatases and provide a specific platform for coordinated phosphorylation-dephosphorylation reactions. John Scott (Portland, OR) reported that AKAP79 assembles two kinases, PKA and PKC, plus the calcium-dependent phosphatase PP2B, on the microtubules, and AKAP220 directs PKA and protein phosphatase 1 (PP1) to the

peroxisomal vesicles. He also suggested that there might be a physical interaction of the AKAP79-mediated regulatory complex with the cAMP generating system and that this might be mediated by direct contact between this complex and the β -adrenergic or glutamate receptors. On the other hand, AKAP149 targets the PKA kinase and PP1 phosphatase to the nuclear envelope (Philippe Collas, Oslo, Norway) to regulate nuclear reassembly at the end of mitosis. Another interesting kinase-binding protein was introduced by Brian Hemmings (Basel, Switzerland), who described a so-called C-terminal modulator protein (CTMP) of protein kinase B/Akt. Overexpression of this protein alters cell morphology and reduces the transforming efficiency of viral Akt, indicating a role for CTMP as a novel tumor suppressor.

Regulatory or targeting proteins that interact with the catalytic subunit of PP1 were the focus of several presentations. Tim Haystead (Durham, NC) presented an effective protein purification and identification method based on affinity chromatography and peptide sequencing. This was used to identify the smooth muscle myosin targeting protein, MYPT1, which localizes PP1 to the myofibers and modulates its activity. Patricia T.W. Cohen (Dundee, UK) compared three glycogen-binding PP1 subunits G_L , R5 and R6, and convincingly demonstrated that, despite the sequence similarities, these proteins exhibit distinct tissue distribution and different regulatory properties. The nuclear inhibitor of PP1 (NIPPI) directs it to the nucleus and Mathieu Bollen (Leuven, Belgium) showed that, besides the phosphatase binding site, this protein also possesses RNA-binding and forkhead-associated domains. The latter regulates pre-mRNA splicing, a function that seems to be independent of PP1 binding. According to Roger Colbran (Nashville, TN), the neuron-specific neurabin I and the more widely expressed neurabin II (or spinophilin) proteins form complexes with the γ isoform of PP1. Spinophilin also binds F-actin and the third intracellular loop of the α_2 adrenergic receptor. The latter multiprotein complex may be uniquely adapted to provide adequate regulation of synaptic

Fig. 2. Some of the possible protein partners interacting with Ser/Thr protein phosphatases are schematically shown in a hypothetical eukaryotic cell. AMPA receptor, glutamate receptor isoform; AKAP, A-kinase anchoring protein; PKA, protein kinase A; PKC, protein kinase C; PP, protein phosphatase; G_L , glycogen-binding subunit of PP1; Bcl-2, B-cell lymphoma protein 2; NIPPI, nuclear inhibitor of protein phosphatase 1; S6K, S6 ribosomal protein kinase; α_2 receptor, α_2 adrenergic receptor. Drawn by John Scott.

functions. However, the physiological significance of the complex needs to be substantiated by additional experiments. Angus Nairn (New York, NY) proved that cdk5 influences dopamine signaling by phosphorylating, and thereby attenuating the inhibitory potential of, the neuronal PP1 inhibitor DARPP-32. This phenomenon may play a role in the adaptive responses of the dopamine system to psychoactive drugs such as cocaine. The growth arrest and DNA-damage-inducible protein GADD34 was found to bind both the phosphatase regulatory protein inhibitor-1 (I-1) and the catalytic subunit of PP1 (Shirish Shenolikar, Durham, NC). This novel ternary complex was detected in brain extracts and was suggested to regulate protein synthesis. Alphonse Garcia (Paris, France) reported on the interaction of the catalytic subunit of the PP1 α isoform with the pro-apoptotic oncogene Bad *in vitro* and *in vivo*. Garcia further demonstrated that the anti-apoptotic protein Bcl-2 also interacts with PP1 α . Disruption of the Bcl-2/PP1 α complex decreases Bcl-2 and Bad-associated phosphatase activity, suggesting that PP1 α may control apoptosis. Although some of the above-mentioned protein-protein interactions are tentative, it became obvious that PP1 has a wide range of functions that is regulated by a myriad of subunits and interacting proteins.

New evidence for the localization and redistribution of the supposedly cytosolic protein phosphatase 2A (PP2A) was presented in four lectures. Estelle Sontag (Dallas, TX) described the co-localization of the PP2A holoenzyme AB α C isoform with junctional proteins, and therefore suggested a role for this enzyme in tight junction formation in epithelial cells. Alistair Sim (Callaghan, Australia) reported that trafficking of PP2A in mast cells might be related to the regulation of histamine secretion. While the B regulatory subunit of PP2A interacts with junctional proteins, the A regulatory subunit binds axin (Jozef Goris, Leuven, Belgium), which brings PP2A into a protein complex involved in Wnt signaling. Together with glycogen synthase kinase 3 β , PP2A regulates β -catenin stability, and thus early embryonic development, in *Xenopus laevis*. In addition, Marc Mumby (Dallas, TX) described a novel PP2A regulatory subunit termed PR48. This subunit binds to the Cdc6 protein and targets PP2A to the DNA replication machinery. The control of replication via the modulation of the pre-replication complex may be an exciting new function of PP2A.

During the meeting, the modular nature of protein interactions emerged as a general organizing principle. Several domains of the same protein may have quite diverse and sometimes independent functions. Thus, a single polypeptide chain could integrate various inputs and be responsible for combinatorial regulation. Research interest will no doubt shift more towards the conceptual building of protein networks from several starting points, which may ultimately link all proteins in the future.

Transgenic surprises

Functional and genetic analyses confirm and extend existing biochemical models and occasionally challenge older hypotheses. A number of presentations demonstrated that knockout and transgenic organisms can be instrumental in identifying sometimes unexpected functions of PTP and PPP family members.

PTP1B is considered to be the main phosphatase to regulate the insulin receptor. PTP1B knockout mice are hypersensitive to

insulin and resistant to obesity. Ben Neel (Boston, MA) explained the latter finding by the involvement of PTP1B in the leptin-based body-weight-controlling pathway. Gab2 is a scaffolding protein that binds Shp-2, another PTP. A detailed analysis of Gab2 knockouts, also presented by Neel, indicated that this protein is an essential regulator of the allergic response. PTP1B and T-cell PTP (TC-PTP) are structurally related non-receptor-type PTPs. Michel L. Tremblay (Montreal, Canada) used cell lines derived from knockout mice to identify the function of PTP1B in integrin signaling and that of TC-PTP in cell cycle regulation. Although these phosphatases are known as negative regulators of growth hormone induced signals, they were found to behave as positive regulators in the aforementioned two pathways. Another PTP found to work in surprising ways was PTPe. Mice in which this gene is knocked out are viable, but, when crossed with mice expressing the active Neu oncogene which readily develop mammary gland cancer, produce offspring in which the rate of tumor formation is significantly reduced. This effect is primarily due to the reduction of Src kinase activity in the knockout mice (Ari Elson, Rehovot, Israel). Thus, it is possible that manipulation of PTPe could be used to slow down cancer progression. The receptor-like PTP CD45 is essential in T-cell receptor signaling. According to Denis Alexander (Cambridge, UK), the stimulation of the CD28 co-receptor in a CD45 minus cell line was down-regulated due to the reduced activity of the Lck kinase. He also reported on functional differences between distinct CD45 isoforms. These results are interesting, since the expression of CD45 isoforms is regulated developmentally.

The binding of PP1 to protein-glycogen particles via its R_{GL} subunit in skeletal muscle has been well documented. Contrary to the expectations based on biochemical studies, however, knockout mice revealed that this subunit is not involved in either insulin or adrenalin action. Instead, it is important in the regulation of glycogen degradation mediated by exercise or neuronal stimuli, as was shown by Anna DePaoli-Roach (Indianapolis, IN). In another example, the phosphorylation of tau protein was studied in a mouse model of Alzheimer's disease (Jürgen Götz, Zürich, Switzerland). Transgenic mice with a dominant negative mutant form of PP2A C α were constructed and used to show that reduction of PP2A activity in the brain results in partial relocation of abnormally phosphorylated tau from the axonal to the somatodendritic compartment. Thus, PP2A plays a critical role in the formation of neurofibrillary lesions associated with the disease. Knockout mice are not the only useful systems for the functional analysis of proteins. Indeed, *Saccharomyces cerevisiae* has also served as an excellent model for the study of phosphatase function. In this organism, two novel phosphatases, Ppz1 and Sit4, share a common regulatory subunit, Hal3. Joaquin Arino (Barcelona, Spain) constructed a Sit4/Hal3 conditional double mutant and unexpectedly found that Nha1, a Na⁺-H⁺ antiporter, rescued the G₁/S cell cycle blockade in the mutant. It was shown that even a small domain of the antiporter protein (not related to its pumping activity) was sufficient for suppressing the cell cycle defect. This may be the first step towards the discovery of a novel cell cycle regulatory mechanism. The yeast and the mouse knockout studies described here clearly demonstrate the power of genetic approaches in the identification of protein phosphatase functions in a complex biological context.

Entering the arena of rational drug design

Tumor viruses utilize protein kinase or phosphatase subunits with deadly effects, and many other organisms have evolved natural phosphatase inhibitors that act as elements of effective protective mechanisms. Cyclosporins were identified fortuitously as immunosuppressors even before their ability to inhibit PP2B was recognized. Now, with our extensive molecular knowledge, the field is ripe for a more rational approach to drug design.

Although protein phosphatases may be promising targets for therapy, investigations in this area are still in their infancy. The X-ray structures of PTP-SL (Stefan Szedlacsek, Bucharest, Romania), PTP1B complexed to an insulin receptor-derived peptide (David Barford, London, UK) and PP1 associated with the natural toxin okadaic acid (Charles Holmes, Edmonton, Canada) were presented at the meeting. These data will certainly aid in the design of novel specific phosphatase inhibitors.

Sara Lavi (Tel Aviv, Israel) showed the potential use of PP2C α itself as an anticancer biodrugg. Overexpression of PP2C α causes irreversible growth arrest at the G₂/M phase transition, thereby inducing apoptosis. PP2C-transfected cells fail to produce tumors, and treatment of tumor-bearing mice by the *N*-(2-hydroxypropyl)methacrylamide co-polymer coupled to PP2C significantly reduced tumor growth rate. Josef Krieglstein (Marburg, Germany) provided a possible molecular mechanism for the above data. It is known that the function of the pro-apoptotic protein Bad is regulated by reversible phosphorylation: growth factors inactivate Bad by inducing its phosphorylation, dissociation from Bcl-X_L and sequestration in the cytosol upon association with the 14-3-3 proteins. Deprivation of survival factors induces Bad dephosphorylation, resulting in apoptosis. Krieglstein showed that Bad is a substrate for PP2C, thus implicating PP2C as a novel regulator in the apoptotic pathway.

The real breakthrough in therapeutic applications has occurred in the kinase field. Several kinase-directed drugs are under clinical tests and one of them has been approved recently for medical use. Some of the current approaches were described in three talks. The three-dimensional structures of the α catalytic subunit of casein kinase 2, both in the presence and in the absence of ATP and inhibitors, have been determined by X-ray crystallography (Lorenzo Pinna, Padova, Italy). Structural data in conjunction with site-directed mutagenesis were used to map the catalytic pocket in precise detail and will lead to the development of more effective inhibitors. Prevention of angiogenesis is a promising strategy in cancer therapy, and it can be achieved by blocking the vascular endothelial growth factor receptor Tyr kinase. Dieter Marmé (Freiburg, Germany) reported that the PTK787 compound selected from a chemical library of 800 000 molecules is highly specific for the kinase and is in phase I of clinical evaluation. Sir Philip Cohen (Dundee, UK) elegantly summarized the field in his keynote lecture. He explained that most of the inhibitors used to date have been ATP analogs that block the active site of the kinases. Some of them have a surprisingly high specificity due to the unique conformations of the catalytic pockets. He selected glycogen synthase kinase 3 (GSK3) as a critical target and announced the development of a new generation of oligopeptide drugs that may specifically

inhibit the phosphorylation of some protein substrates without affecting the phosphorylation of other proteins. These drugs would be able to mimic insulin treatment without any unwanted side effects.

The above examples indicate that the therapeutic potential of kinases will certainly be further exploited and suggest that, in the future, phosphatase-directed drugs could also be introduced into medical practice.

An uncharted territory

To date, kinases and phosphatases in eukaryotes have been associated almost exclusively with the modification of Ser, Thr and Tyr residues. Scientists working with prokaryotes already knew there was more to the story. Jeffrey B. Stock (Princeton, NJ) reviewed bacterial 'two-component systems' consisting of a His kinase sensor molecule plus a corresponding response regulator. These His kinase catalytic domains are unlike those of the Ser/Thr or Tyr kinases and are, in fact, more closely related to ATPase domains. Harry Matthews (Davis, CA) summarized what is known about homologs of the bacterial His kinases in yeast, fungi, slime mold and plants.

There are important examples of phosphohistidine in mammalian proteins (e.g. G-proteins, P-selectin and annexin). Although corresponding mammalian His kinases still remain an enigma, a PHP has been discovered in vertebrates (Susanne Klumpp, Marburg, Germany). Its primary structure and insensitivity to known inhibitors indicate that this novel His phosphatase is unlike any of the known Ser/Thr or Tyr phosphatases. Interestingly, PHP has been shown to be present in animals ranging from humans to nematodes but absent in bacteria.

This first session on phosphorylation and dephosphorylation of His residues revealed that a critical mass of information has been collected in recent years. It also became obvious that the role of His phosphorylation and dephosphorylation in mammalian cells is only just beginning to be explored.

Conclusions

In theory, one kinase and one phosphatase can operate a regulatory cycle. In practice, however, a multitude of cycles are intimately interrelated through overlapping substrates and, more importantly, through the mutual regulation of kinases and phosphatases by other kinases and phosphatases. Thus, the key regulators of cellular responses are themselves under the control of yet another set of regulators. These regulatory cycles possess an enormous capacity for amplification, integration and processing of internal and external signals. They play a significant role in virtually all aspects of eukaryotic cellular functions. Even a small defect in the signaling network may result in serious regulatory disorders. A direct connection between impaired signaling and disease is becoming more and more appreciated and, in recent years, these interconverting enzymes have become prime targets of rational drug design. The Marburg meeting highlighted the central role and the potential applications of the phosphatases and kinases through several stimulating discussions.

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