The ins and outs of protein phosphorylation

Workshop report: Control of signaling by protein phosphorylation

The Centre for International Meetings on Biology was established in 1992 as a component of a private foundation: Fundación Juan March. Every year, it organizes a range of workshops and scientific activities covering different aspects of modern biology. At these meetings, scientists actively involved in the different fields present their latest results in a meeting format with about 22 invited speakers and a relatively small number of participants. This promotes discussion and a rich exchange of results and ideas.

of receptor tyrosine kinases in regulating cell growth, proliferation and survival. An illustration of how these pathways are thought to branch from the receptor kinase, and the output of each branch is shown in Figure 1.

The role of the PI3K pathway in mitogenic signaling was discussed by several speakers. T. Hunter (La Jolla, CA) showed that mice harboring a c-Kit receptor point mutation that prevents docking and activation of PI3K, but does not affect signaling mediated by other effectors, had normal hematopoiesis and pigmentation, although these processes are aberrant in c-Kit-deficient mice. Instead, loss of PI3K activation alone resulted in defects in spermatogonial stem cell survival. These results indicate that the PI3K-mediated component of c-Kit signaling is essential for effecting only a subset of c-Kit-mediated responses. L. Cantley (Boston, MA) discussed the role of PI3K in insulin signaling based on experiments in which the mouse p85 α subunit of PI3K was deleted. Mice deficient for this protein mostly died at birth, but outbreeding the few surviving mice resulted in 20–30% viability. Such mice were hypoglycemic and had low levels of circulating insulin, but were hypersensitive for glucose uptake and Glut4 translocation in peripheral tissues. Cantley suggested that this hypersensitivity could result from breaking a negative feedback loop that might normally desensitize insulin receptor substrates (IRSs), the link between the insulin receptor and PI3K.

Further downstream along the PI3K pathway lie phosphoinositide-dependent protein kinase (PDK)-1, protein kinase B (PKB, also known as Akt) and p70 ribosomal S6 kinase (S6K)-1. Several talks focused on the relationships among these molecules. G. Thomas (Basel, Switzerland) presented data from a genetic screen in *Drosophila*, which his group is using to search for effectors of S6K1. Results from their studies are consistent with the idea that S6K1 acts independently of PKB, with the two proteins representing a bifurcation of the PI3K pathway downstream of PDK1 (Figure 1). PDK1 has been proposed to be a key regulator of ribosomal subunit kinase (RSK) proteins as well as of S6K1 and PKB. The report by P. Cohen (Dundee, UK) that mouse ES cells homozygous mutant for PDK1 are viable and have normal growth rates was therefore unexpected, especially in light of the fact that PKB, S6K1 and all RSK family proteins fail to become activated in these cells. These cells showed robust CREB phosphorylation in the absence of RSK (shown to be the CREB kinase in other systems) activity, whereas ES cells lacking mitogen- and stress-activated protein kinase (MSK)-1 failed to phosphorylate CREB, even in the presence of active RSK. It therefore appears that in this system MSK1, rather than RSK, is

Introduction

Dissecting the interrelationships among components of the molecular signaling pathways involved in the control of key physiological processes such as cell growth, differentiation, survival and development is a timely issue in biology. The activation of these pathways is generally triggered at the plasma membrane by specific agonists acting through their cognate receptors. The activated receptors in turn initiate signaling cascades that ultimately control the activities of specific intracellular effectors. Much of the regulation of these pathways is achieved by manipulating the phosphorylation states of various components of these cascades. The kinases and phosphatases that mediate this control were the topic of a recent Juan March Workshop held in Madrid (March 13–15, 2000). It became clear during this meeting that the vast amounts of data generated from cell culture experiments must be validated in more complex *in vivo* systems to determine the contribution of each signaling pathway to a given function.

Mitogenic and survival signaling

Many of the talks at this meeting revolved around the roles of the phosphoinositide-3-kinase (PI3K) and mitogen-activated protein kinase (MAPK) signaling pathways that participate downstream

Fig. 1. Pathways controlling cell cycle progression and survival. Two signaling cascades emanate from the IRS molecules. One may signal survival, cell growth, and cell cycle progression through the PI3K branch and another, which constitutes a cooperative or overlapping pathway, operates through the MAPK cascade. The former can be down-regulated by the lipid phosphatase PTEN and acts independently of the MAPK pathway. Its effects on S6K1 regulate the translational machinery, influencing cell growth and cell division. The MAPK pathway may use different CREB kinases in a context-specific manner.

the CREB kinase. Whether or not these results simply reflect cell type specificity awaits further study. Consistent with prevailing knowledge of the PI3K pathway, MAPK signaling was unaffected in the PDK1-deficient ES cells.

In contrast to the data presented above, evidence presented by E. Hafen (Zürich, Switzerland) implied cross-talk between the MAPK and PI3K pathways. A modifier screen in *Drosophila* identified a line overexpressing PDK1 as a suppressor of phenotypes generated by mutations that upregulate the MAPK pathway. Hafen went on to report that flies deficient for *dPDK1* are smaller in size, as is the case for the IRS loss-of-function phenotype previously described by Hafen's group. Hence, PDK1 seems to have some role in growth regulation.

The presentation of M. Cobb (Dallas, TX) focused on the MAPK branch of the pathway. She reported on the effects of constitutively active mutants of one MAPK, the extracellular signal-regulated kinase (ERK)-2, in pancreatic β cell signaling. Activated ERK2 induces insulin transcription, whereas dominant-negative variants of ERK2 or specific inhibitors of the MAPK signaling pathway block this response. Using these tools, Cobb's group identified Beta2/NeuroD1, a transcription factor known to bind to the insulin promoter, as a direct ERK2 target involved in glucose-induced insulin transcription. E. Van Obberghen (Nice, France) reported that the activated insulin receptor recruits the

protein signal transducer and activator of transcription (Stat)-5b, in addition to its other targets. This recruitment leads to activation and nuclear translocation of Stat5b, and induction of a number of genes containing Stat5b recognition motifs. In parallel, Stat5b activates suppressor of cytokine signaling (SOCS)-3, which binds to the same site on the insulin receptor, potentially providing an elegant feedback mechanism for regulating insulin signaling. F. de Pablo (Madrid, Spain) reported that regulated expression of proinsulin mRNA occurs prior to the expression of insulin-like growth factor (IGF)-1 during chick embryo neurulation and retinal neurogenesis. She went on to explain that insulin/IGF-1 hybrid receptors bind proinsulin and insulin with high affinity during early neural development, and treatment with antisense oligonucleotides or antibodies against proinsulin/insulin or the insulin receptor, but not against IGF-1, results in increased apoptosis in the locations in the neuroretina or the neurulating embryo where it occurs naturally. Together, these findings demonstrate that insulin, most likely in the form of proinsulin, signals prior to IGF-1, in survival pathways used during development.

C. Marshall (London, UK) discussed the connection between cell cycle progression and early mitogenic signaling. He reported that the lack of the cell cycle regulator pRb105 significantly reduces the requirement for ERK during S-phase entry,

Fig. 2. Different signaling adapters involved in IKK and NF-κB activation. The signaling pathways that activate NF-κB in response to IL-1 or TNF-α involve functionally similar proteins. The TNFR1 recruits TRADD and TRAF2, along with RIP, which interacts with the inhibitory protein RIP3 and the adapter p62. The activation of IL-1 leads to the recruitment of MyD88 and IRAK, which together with TRAF6 are essential components in the activation of the IKK signaling complex. The IKK signaling complex can also be activated by PMA, most probably through a classical PKC isoform such as αPKC, and by dsRNA through PKR.

suggesting a role for this MAPK in the activation of cyclin D-dependent kinases (cdks) that phosphorylate and inactivate pRb105. Furthermore, blocking the ERK pathway inhibits the activation of cdk4–cyclinD1 complexes, but not of cdk2–cyclinE complexes. The cell cycle was also covered by M. Barbacid (Madrid, Spain), who reported that mice deficient for cdk4 develop insulin-dependent diabetes. In contrast, those harboring activated alleles overproduce insulin. However, these defects are probably not directly related to insulin signaling, but rather result from proliferation defects during pancreatic development.

J. Downward (London, UK) focused on the role of the MAPK pathway in cell survival. He showed that apoptosis induced by either transforming growth factor (TGF)-β or tumor necrosis factor (TNF)-α can be blocked by c-Raf. The precise mechanism whereby c-Raf is able to block apoptosis induced by such different stimuli remains unclear, but it must affect a common step in the two pathways. These studies further suggest that ERK, which is downstream of c-Raf, is an anti-apoptotic kinase, as has long been proposed by others.

The above constitutes a large body of complex data. However, an important generalization emerges from it. In different cell systems, mitogenic signaling through a single receptor type can be mediated by different pathways acting in concert. The variations between systems may reflect context specificity, or possibly feedback loops. Studies combining vertebrate animal

models such as knock-out mice with genetically simpler systems like *Drosophila* and *Caenorhabditis elegans*, as well as experiments in cell lines, will provide a better understanding of the real contribution of each pathway.

Cytokine signal transduction

Various talks centered on paradigms for cytokine signaling, with many focusing on the regulation of NF-κB activation. Figure 2 illustrates how a number of signaling components and adapters are thought to regulate the phosphorylation and degradation of IκB to release NF-κB from its sequestered, cytosolic state, ultimately allowing for its nuclear translocation. The kinase that phosphorylates IκB (IKK) consists of two catalytic subunits (IKKα and IKKβ) and an essential adapter protein (IKKγ or Nemo).

M. Karin (La Jolla, CA) showed data that suggest that IKKα is not essential for IKK activity. IKK activity is intact in mice deficient for IKKα, whereas the activation of both IKK and NF-κB is abolished by the deletion of IKKβ. Indeed, the phenotype of IKKβ-deficient mice resembles that of mice deficient for RelA, a critical component of the NF-κB complex. Karin went on to show that the double-stranded RNA activated kinase, PKR, is able to activate IKK *in vitro* and *in vivo* even when devoid of enzymatic activity. This raises the possibility that phosphorylation of IKK may not be necessary for its activation. J. Moscat

(Madrid, Spain) reported that the overexpression of ζ -protein kinase C (ζ PKC) is sufficient to activate IKK β . However, in contrast to activation by PKR, activation by ζ PKC is phosphorylation dependent, as a ζ PKC kinase-inactive dominant interfering mutant severely impairs IKK β activation. Moscat further showed that the ζ PKC-interacting protein, p62, is required for the activation of NF- κ B by TNF- α and interleukin (IL)-1. The activation of IKK by TNF- α was inhibited in p62-deficient cells, whereas ERK activation by epidermal growth factor (EGF) was unaffected, demonstrating that p62 activation has a restricted target range and may be specific to the NF- κ B pathway. Interestingly, p62 binds to both receptor interacting protein (RIP) and tumor necrosis factor receptor-associated factor (TRAF)-6, two important intermediaries of TNF and IL-1 signaling, respectively. These interactions allow the two pathways to feed into NF- κ B signaling. V. Dixit (San Francisco, CA) discussed another level of control that is mediated by RIP3, a RIP homolog which is a potent inhibitor of NF- κ B activation. RIP3 interacts with the intermediary domain of RIP, and might thereby displace an effector of the NF- κ B pathway, perhaps p62. The fact that PKR does not require its enzymatic activity to activate IKK and that it can dimerize in response to dsRNA, suggests that oligomerization of the different adapters may be sufficient to trigger activation of the IKK complex. Indeed, RIP, TRAF6, p62 and the atypical PKCs all have the capacity to dimerize and to form large oligomeric complexes. The IL-1 receptor-associated kinase (IRAK) and RIP are also examples of kinases that activate NF- κ B independently of their enzymatic activity.

The theme of cytokine signaling was continued by J. Ihle (Memphis, TN), who discussed erythropoietin (Epo) signaling through the Janus kinase (JAK)–Stat5 pathway. While JAK2-deficient mice are embryonic lethal and exhibit failure of fetal liver erythropoiesis, Stat5a/b-deficient mice were found to have normal erythropoiesis in spite of exhibiting other defects. Potential roles of the proto-oncogene *myc* and the anti-apoptotic factor Bcl-XL as downstream targets of JAK2 were explored. The findings that Epo fails to induce Bcl-XL in JAK2-deficient cells, and that ectopic expression of Bcl2 in JAK2-deficient cells blocks apoptosis and rescues fetal liver erythropoiesis in JAK2-deficient mice, suggest that JAK2 normally induces Bcl-XL in a Stat5-independent manner.

The presentation by Ihle is a nice example of how the combination of different *in vivo* animal models gives the most definitive characterization of the relative roles of different signaling components in a given process. Regarding the NF- κ B pathway, it is clear that different signaling proteins must be recruited to the TNF receptor complex to activate the IKK conglomerate. A key issue now is the identification of the proximal cause of IKK activation. Whether this relies on kinase activation, on the multimerization of adapter proteins, or on a combination of both events awaits further studies in which various knock-out mice are crossbred, and endogenous signaling complexes analyzed in cytokine-activated cells derived from these animals.

Development and differentiation

The theme of developmental biology was the central issue in the following presentations. The first, by M. Greenberg (Cambridge,

MA), focused on a primary cerebellar granule cell culture system used to analyze the role of ephrin B receptors in the formation of excitatory synapses. Oligomerized, soluble ephrin (Eph) B1 stimulated Eph B2 receptor clustering, followed by NMDA receptor-1 clustering. This stimulatory effect depends on the extracellular domain of the Eph B2 receptor rather than on kinase activity, since a kinase-dead receptor or even a construct lacking the cytoplasmic domain still induced clustering. However, receptor kinase activity was not entirely dispensable for the function of this molecule, as it was still found to be essential for promoting synapse formation. This example illustrates the importance of understanding the systems that are used to tease apart complex signaling pathways well enough to design the appropriate assays for testing a given hypothesis.

Eric Wieschaus

Another talk that highlighted the central importance of phosphorylation to signaling processes involved in developmental regulation was presented by E. Wieschaus (Princeton, NJ). Wieschaus discussed a genetic approach for the identification of zygotic genes required for the mid-blastula transition during *Drosophila* embryogenesis. The main characteristic of this process is the ordered cellularization of a multi-nuclear syncytium. A systematic screening of embryos deficient for chromosomal segments identified a gene encoding a protein kinase whose expression must be downregulated for the mid-blastula transition to occur. Wieschaus also discussed work on the Armadillo (Arm)/ β -catenin pathway. In *Drosophila*, Arm functions both as a component of the adhesive junctions and as a transcription factor. Its levels are upregulated by Wnt signaling and its vertebrate homologs are downregulated by the adenomatous polyposis coli protein (APC). The nuclear accumulation of Arm plays an important role in mediating Wnt signaling. Again employing a genetic approach, Wieschaus has identified two *Drosophila* homologs of APC that affect the nuclear localization of Arm.

Novel structure–function relationships

At the molecular level, kinases and phosphatases share many common attributes. However, they also have distinct properties that explain their selective modes of action. S. Harrison (Cambridge, MA) explored this issue by comparing the X-ray structure of the Ser/Thr kinase p21-activated kinase (Pak)-1 with that of Src. In both proteins, the inactive state is characterized by regions of the polypeptide chain outside of the kinase domain stabilizing a conformation of the small lobe of the kinase that disrupts the catalytic site. In Src, an SH2 domain binds to a tyrosine residue in the C-terminal tail of the protein, and an SH3 domain binds to a proline-containing linker region connecting the SH2 domain to the protein tyrosine kinase core. In Pak1, the kinase domain is maintained in an inactive state by dimerization of CRIB domains. This CRIB domain is regulated by Pak1 activators and, consistently, its structure is similar to

that of the corresponding region in WASP, which leads to regulation by its binding partners.

J. Dixon (Ann Arbor, MI) turned the discussion to phosphatases with a talk overviewing the recent structural analysis of the tumor suppressor protein PTEN. This protein functions as a phosphatase for 3-phosphorylated phosphoinositides such as PtdIns(3,4,5)P₃ (PIP₃). Variation in the levels of PTEN expression and changes in its phosphatase activity regulate PKB function and ultimately control cell survival. Dixon described the crystal structure of PTEN, comparing its active site to those of protein tyrosine phosphatases and dual-specificity phosphatases. He explained that the active site of PTEN is modified to accommodate the lipid moiety of PIP₃. Structures like that presented here provide a general model for understanding the specificities of phosphatases towards their cellular substrates.

J. Schlessinger (New York, NY) focused on critical events upstream of kinases and phosphatases that set the stage for their activation. In one example, he described the crystal structure of fibroblast growth factor (FGF)-2 in complex with the extracellular ligand binding domain of FGF receptor (FGFR)-1. This structure shows that FGF has primary and secondary binding sites for FGFR, and that receptor dimerization is stabilized by both receptor-receptor interactions and the binding of heparin sulfate proteoglycans to a positively charged canyon of exposed basic residues. This structural information may provide a framework for understanding the deleterious effects of FGFR mutations that lead to numerous forms of human skeletal disorders.

Conclusion

In conclusion, this meeting addressed the most recent evidence regarding the roles of kinases and phosphatases, and the mechanisms whereby they function during cell signaling. It is becoming increasingly apparent that much redundancy and cross-talk exists between separate pathways. Also, there is some evidence that enzymatic activity may not always be necessary for a particular kinase protein to carry out all of its functions. The use of genetic models, including mutant and transgenic mice and flies, is helping to determine which of the known components in the different signaling cascades are essential for a given

function, and is proving useful for finding novel, and often unanticipated, roles for known signaling molecules.

References

- Datta, S.R., Brunet, A. and Greenberg, M.E. (1999) Cellular survival: a play in three Acts. *Genes Dev.*, **13**, 2905–2927.
Edgar, B.A. (1999) From small flies come big discoveries about size control. *Nature Cell Biol.*, **1**, E191–E193.
Hunter, T. (2000) Signaling-2000 and beyond. *Cell*, **100**, 113–127.
Rothwarf, D.M. and Karin, M. (1999) The NF- κ B activation pathway: a paradigm in information transfer from membrane to nucleus. *Science's STKE*, 26 October 1999 (<http://www.stke.org>).

*George Thomas¹, Flora de Pablo²,
Joseph Schlessinger³ and Jorge Moscat^{4,*}*

¹Friedrich Miescher Institute, Maulbeerstrasse 66, 4058 Basel, Switzerland,
²Centro de Investigaciones Biológicas (CSIC), Velázquez 144, 28006 Madrid,
⁴Centro de Biología Molecular 'Severo Ochoa' (CSIC-UAM), Universidad Autónoma, Canto Blanco, 28049 Madrid, Spain and
³Department of Pharmacology and Skirball Institute, New York University Medical School, New York, NY 10016, USA

Received April 5, 2000; accepted May 23, 2000

*Corresponding author. Tel: +34 91 397 8039; Fax: +34 62 969 0055;
E-mail: jmoscat@cbm.uam.es

DOI: 10.1093/embo-reports/kvd014