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The Role of Protein Phosphatases in the Regulation of Mitogen and Stress-activated Protein Kinases

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It is now established that a family of dual-specificity protein phosphatases are able to interact with mitogen and stress-activated protein kinases in a highly specific manner to differentially regulate these enzymes in mammalian cells. A role for these proteins in negative feedback regulation of MAP kinase activity is also supported by genetic and biochemical studies in yeasts and *Drosophila*. More recently it has become clear that other classes of protein phosphatase also play key roles in the regulated dephosphorylation of MAP kinases, including tyrosine-specific protein phosphatases and serine/threonine protein phosphatases. It is likely that a complex balance between upstream activators and these different classes of MAP kinase specific phosphatase are responsible for determining, at least in part, the magnitude and duration of MAP kinase activation and hence the physiological outcome of signalling.

Keywords: MAP kinase, phosphatase, stress, signal transduction

THE MAP KINASE FAMILY OF ENZYMES

Mitogen-activated protein (MAP) kinases constitute a family of closely related serine/threonine protein kinases which are key components of highly conserved signal transduction pathways.[1–3] These pathways serve to relay, amplify and integrate diverse signals and allow the cell to co-ordinate a wide variety of cellular functions. These include proliferation, differentiation, development, inflammatory responses, growth arrest and apoptosis. As many as eleven MAP kinase family members have thus far been identified in mammalian cells (Figure 1) and these have been classified according to their differential activation by various agonists.[4]

The most widely studied of the MAP kinases are the "classical" 42- and 44-kDa isoforms (also known as ERK2/ERK1 or MAPK2/MAPK1) which respond vigorously to growth factors and phorbol esters and have been associated with cellular proliferation and differentiation.[5] A second group of kinases are preferentially activated by cellular stress including oxidative stress, osmotic shock, DNA-damaging agents and inhibitors of protein synthesis. These comprise the stress-activated protein (SAP) kinases and include the c-Jun kinase isoforms JNK-1, JNK-2

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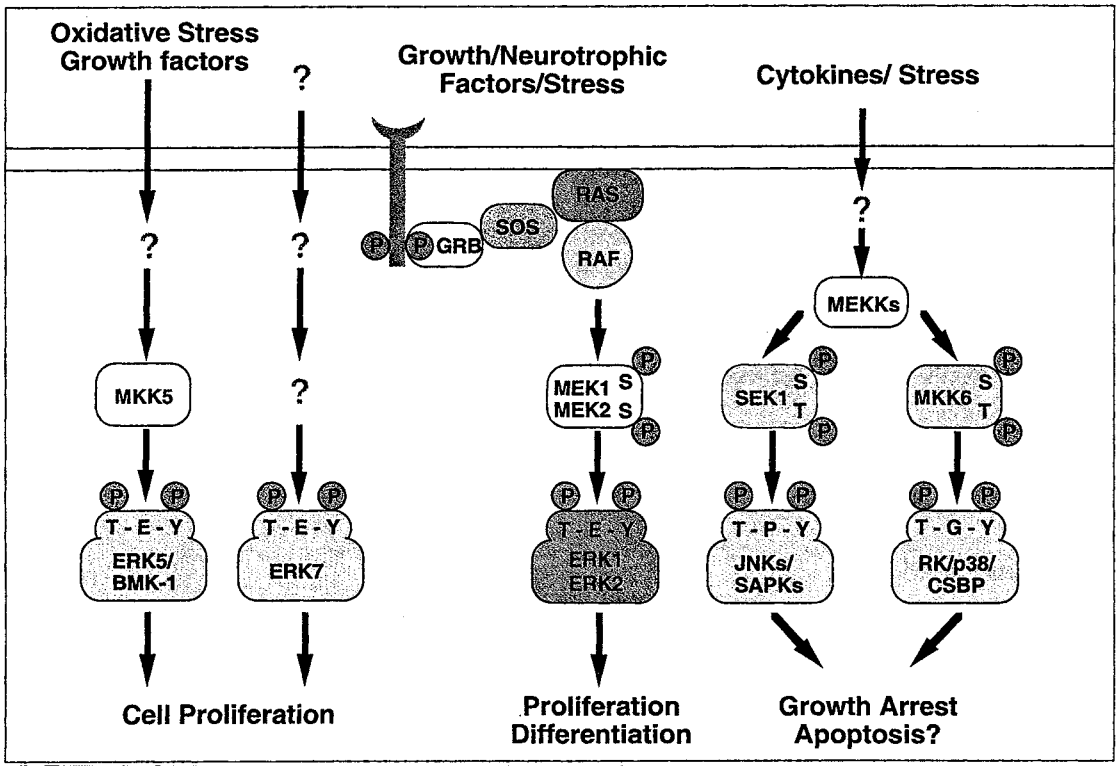

FIGURE 1 *Simplified overview of MAP and SAP kinase signalling cascades in mammalian cells.* The "classical" MAP kinase cascade containing the ERK1 and ERK2 MAP kinases is shown lying downstream of growth factor receptors and ras and its activity is associated with stimuli which cause cell proliferation or differentiation. Recently two other MAP kinase have been characterised which also contain the signature sequence T-E-Y. These are BMK-1/ERK5 and ERK7. Of these, BMK-1/ERK5 has recently been shown to be activated by both oxidative stress and growth factors and to induce the expression of immediate early genes by targeting the transcription factor MEF2C. The function of the ERK7 MAP kinase and its mechanism of activation are as yet unclear. Two known stress activated MAP kinase cascades are shown. The JNKs or SAPK1 enzymes which contain the signature sequence T-P-Y and the p38 or SAPK2 enzymes which contain the signature sequence T-G-Y. The activity of these pathways has been associated with stress induced growth arrest and apoptosis in certain cell types.

and JNK-3 (SAPK1c, SAPK1a and SAPK1b), p38/RK/CSBP (SAPK2a), p38β (SAPK2b), ERK6/P38γ (SAPK3) and SAPK4.[4] More recently, two additional MAP kinases have been identified. These are ERK5/BMK1[6,7] (also known as SAPK5) and ERK7.[8] While it is clear that MAP and SAP kinases may phosphorylate a number of cytosolic targets in mammalian cells, much of the excitement which has surrounded the characterisation of these enzymes has been generated by the finding that transcription factors are major targets for these pathways. It is now clear that MAP kinase signalling constitutes a major route for the transmission of information from cell surface receptors to the nucleus. Furthermore, the transcription factors which are the targets of MAP and SAP kinase signalling include several gene products implicated in the control of cellular growth and responses to environmental stress.[4,9]

All MAP kinases are activated by phosphorylation of both threonine and tyrosine residues within the conserved signature sequence T-X-Y by a dual specificity MAP kinase kinase (MEK or MKK). These enzymes are themselves activated by phosphorylation of serine/threonine residues by a group of MAP kinase kinase kinases (MAPKKKs or MEKKs) which, in the case of ERK1/2, include the raf protein kinase.[2] This

basic arrangement of the MAP kinase module is highly conserved from yeast to man. In addition to mediating activation of MAP kinases, it has recently been demonstrated that phosphorylation of the both the threonine and tyrosine residues within the T-X-Y signature sequence is responsible for promoting the dimerisation and nuclear translocation of ERK2/MAPK2.[10] Other MAP and SAP kinases also form dimers, indicating that this is likely to be a general mechanism for nuclear translocation of these enzymes. This has important functional implications, as the magnitude and duration of MAP kinase signalling, coupled with nuclear translocation of the enzyme are critical determinants of biological effect.[11] This is best illustrated by studies of the differentiation of rat PC12 cells in culture. These cells proliferate in response to epidermal growth factor (EGF) while exposure to nerve growth factor (NGF) causes cell differentiation marked by neurite outgrowth. This differential response is entirely due to the ability of NGF to cause both sustained activation and nuclear translocation of MAP kinase. In contrast, EGF causes only a very transient activation of MAP kinase and the kinase does not enter the cell nucleus.[12]

PROTEIN PHOSPHATASES AND THE REGULATION OF MAP KINASE ACTIVITY

Both the duration and magnitude of MAP kinase activation are likely to be determined by the competing activities of upstream activating kinases (MEKs or MKKs) and protein phosphatases. Phosphorylation of both the threonine and tyrosine residues within the activation sequence T-X-Y is required for MAP kinase activity and dephosphorylation of either residue is sufficient to inactivate the kinase. The latter process could be mediated either by serine/threonine specific protein phosphatases, tyrosine specific phosphatases (PTPases) or by dual-specificity (Thr/Tyr) protein phosphatases. Recent evidence both from mammalian cells and from lower organisms such as yeasts and *Drosophila*, indicates that all three classes of protein phosphatases play a role in regulating the activity of these key enzymes *in vivo*.

The Dual-specificity Map Kinase Phosphatases

The first MAP kinase specific phosphatase to be isolated and characterised was the human CL100 enzyme (also known as 3CH134, *erp* and MKP-1). Expression of CL100 gene is highly inducible by growth factors, oxidative stress and heat shock and the protein is localised within the nucleus when expressed in mammalian cells. CL100 contains a catalytic domain with significant amino acid sequence homology to a dual-specificity protein phosphatase (VH-1) from vaccinia virus[13] and a non-catalytic amino-terminal domain which contains two short regions of sequence homology with the catalytic domain of the cdc25 phosphatase.[14] CL100 (MKP-1) is able to specifically dephosphorylate and inactivate ERK2 MAP kinase both *in vitro* and *in vivo*.[15,16] This latter property coupled with the observation that CL100 expression is induced by many of the stimuli which activate MAP and SAP kinases strongly suggests that CL100 is involved in negative feedback control of MAP kinase activity.

Support for such a role *in vivo* first came from studies of the mating response in *S. cerevisiae*. Haploid yeast cells respond to mating pheromone by activating the Fus3p/Kss1p MAP kinase signalling pathway.[17] Activation of this pathway leads to an initial block in the G1 phase of the cell cycle which is gradually overcome in the continued presence of pheromone. A genetic analysis of this adaptation process by Doi *et al.*[18] led to the isolation of *MSG5* as a suppressor of pheromone-induced G1 arrest. Furthermore, deletion of *MSG5* both diminished the adaptive response and enhanced the pheromone induced phosphorylation and activation of the Fus3 MAP kinase. At the amino acid sequence level *MSG5* is most

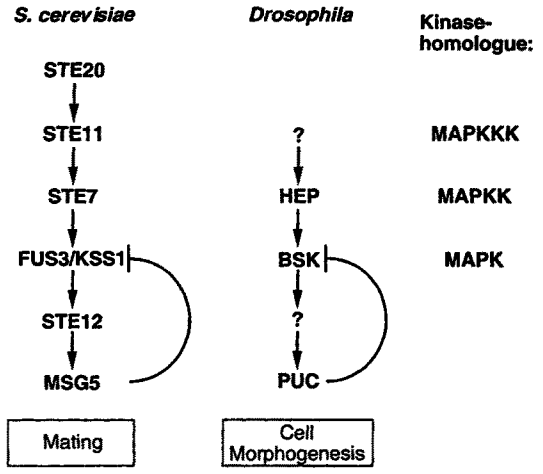

FIGURE 2 *Negative feedback regulation of MAP kinase signalling by inducible dual-specificity MAP kinase phosphatases in S. cerevisiae and Drosophila.* Budding yeast respond to mating pheromone by activating the Fus3p/Kss1p MAP kinases which leads to cell cycle arrest. Fus3p then activates the transcription factor encoded by *STE12* which is responsible for the inducible expression of the dual-specificity phosphatase Msg5p. Msg5p dephosphorylates and inactivates the Fus3p MAP kinase in a negative feedback loop. This feedback inhibition mediates, at least in part, the adaptive response to pheromone. During the process of dorsal closure in *Drosophila* the JNK homologue encoded by *bsk* is activated downstream of its MAP kinase kinase *hep*. Activation of bsk leads to induction of the dual-specificity phosphatase encoded by *puc* which, like Msg5 mediates feedback inhibition of *bsk*. This fine control of *bsk* activity is required for the correct completion of dorsal closure.

similar to CL100 (MKP-1) and Msg5p efficiently dephosphorylates both the threonine and tyrosine residues required for activation of Fus3p. Finally, in support of a role for *MSG5* in negative feedback regulation of this MAP kinase signalling pathway, the expression of *MSG5* is pheromone-inducible and this induction is mediated by the transcription factor Ste12p, which lies downstream of Fus3p in the signalling pathway (Figure 2).

More recently, Martin-Blanco *et al.*[19] have characterised a gene in *Drosophila* called *puckered* (*puc*). When mutated, *puc* affects the process of dorsal closure during embryogenesis. A MAP kinase signalling pathway which contains *basket* (*bsk*), the *Drosophila* homologue of the mammalian Jun kinase (JNK or SAPK1), plays a role in the process of dorsal closure and *puc* was found to encode a CL100-like phosphatase. It was also demonstrated that *puc* has phosphatase activity towards *bsk* and that *puc* expression is dependent on activation of *bsk*. The conclusion is that *puc* encodes a dual-specificity MAP kinase phosphatase which is responsible for negative feedback regulation of *bsk* (Figure 2).

It is now clear that CL100 is prototypic for a family of at least eight mammalian dual-specificity MAP kinase phosphatases (MKPs). Certain of these, including PAC-1, hVH-2 (also known as MKP-2 or Typ-1), and hVH-3 (also known as B23) are, like CL100, nuclear proteins which are encoded by stress and/or mitogen inducible genes.[20] Indeed, until fairly recently, it was assumed that the function of this class of MKPs would be restricted to the cell nucleus. However, other members of this family of proteins have recently been characterised which exhibit quite different properties. These are Pyst1 (also known as rVH-6 and MKP-3),[21–23] Pyst2 (also known as MKP-X)[21,22,24] MKP-4[25] and M3/6 (also known as hVH-5).[26,27]

MKPs can Discriminate between Different MAP Kinase Isoforms

The existence of at least eight mammalian MKPs implies considerable complexity in the regulation of MAP kinase signalling by these proteins. Some of this complexity might be accounted for by tissue specificity of expression. While there is some evidence for the latter in the observation that the expression of the PAC-1 phosphatase is largely restricted to cells of the haematopoetic system, Northern blot analyses have indicated that multiple members of this family of enzymes are expressed in a single cell type.[21] The first clue that certain of the MKPs might behave differently when compared with CL100 came with the characterisation of the Pyst1 and Pyst2 enzymes. These two MKPs, together with a third enzyme designated MKP-4, are more similar in amino acid sequence to each other than to the other MKPs

indicating that they represent a distinct subfamily.[24] Unlike CL100, neither Pyst1 nor Pyst2 are immediate early genes in human skin fibroblasts nor are they inducible by cellular stress. In addition, when these proteins were expressed in mammalian cells they were localised predominantly within the cytosol indicating that different MKPs might act to regulate MAP kinases within distinct subcellular compartments. Recent studies have now revealed that both Pyst1 and M3/6 (hVH-5), another cytosolic MKP, show a remarkable ability to discriminate between different MAP and SAP kinase isoforms both *in vitro* and *in vivo*.

Pyst1 was expressed and purified from bacteria and its activity against different MAP and SAP kinases was determined. In contrast to CL100, which was equally active towards both ERK2 MAP kinase and p38 (SAPK2a), Pyst1 was approximately 100 fold more active towards ERK2 than against this SAP kinase *in vitro* and was also unable to inactivate jun-associated kinases from sodium arsenite treated fibroblasts.[21] Furthermore, when co-expressed with different MAP and SAP kinases in Cos-1 cells, CL100 was able to inactivate ERK2, p38 and JNK1. In contrast, Pyst1 was active towards ERK2 but unable to suppress the activation of the SAP kinases *in vivo* (see Table I). More recent studies have indicated that the other two members of this subfamily, Pyst2 and MKP-4, also exhibit substrate selectivity for ERK.[24,25] In contrast to the members of the Pyst subfamily of MKPs, the mouse M3/6 (also known as hVH-5) enzyme is without activity towards the "classical" MAP kinases ERK1 and ERK2. However, it readily dephosphorylates and inactivates both JNK1 and p38 SAP kinases *in vivo* thus displaying a reciprocal substrate selectivity when compared to Pyst1.[28] As mentioned previously, CL100 can dephosphorylate and inactivate a number of different MAP and SAP kinases. In a more extensive survey of the activities of the inducible, nuclear MKPs Chu *et al.*[29] found that these enzymes showed no marked substrate selectivity but were somewhat less active against SAP kinase isoforms than towards ERK1 and ERK2 MAP kinases.

TABLE I Substrate specificities of the mammalian dual-specificity MAP kinase phosphatases

Enzyme	Localisation	Substrate selectivity	Refs.
CL100/MKP-1	Nuclear	ERKs = JNKs = p38	[29]
PAC-1	Nuclear	ERKs > p38 ≫ JNKs	[29]
hVH-2/MKP-2/ Typ-1	Nuclear	ERKs > JNKs ≫ p38	[29]
hVH-3/B23	Nuclear	Not determined	
Pyst1/rVH-6/ MKP-3	Cytosolic	ERKs ⋙ JNKs = p38	[21,28]
Pyst2/MKPX	Cytosolic	ERKs ⋙ p38 ≫ JNKs	[24]
MKP-4	Cytosolic	ERKs ≫ JNKs = p38	[25]
hVH-5/M3/6	Cytosolic	JNKs > p38 ⋙ ERKs	[28]

Catalytic Activation and a Structural Basis for Substrate Selectivity

In addition to the ability of the Pyst1 to selectively dephosphorylate and inactivate the ERK2 MAP kinase, it was observed that, unlike CL100, wild-type Pyst1 was able to form a tight physical complex with endogenous ERK2 protein when expressed in Cos-1 cells.[21] Furthermore, this binding did not require activation of the MAP kinase. Work by Muda *et al.* has subsequently demonstrated that this binding is mediated by the non-catalytic amino-terminal domain of the Pyst1 (MKP-3) protein *in vitro* and that loss of this binding domain abrogates substrate selectivity *in vivo*.[30] Furthermore, on binding to MAP kinase, Pyst1 undergoes catalytic activation *in vitro* as revealed by a greatly increased activity towards the chromogenic substrate *para*-nitrophenylphosphate (*p*-NPP).[31] Consistent with the substrate selectivity of Pyst1, SAP kinases did not bind to the phosphatase or increase the activity of the enzyme *in vitro*. The closely related enzymes Pyst2 and MKP-4 also exhibit catalytic activation on binding to ERK2 and, like Pyst1, the ability of a range of different MAP and SAP kinases to cause an increase in the rate of hydrolysis of *p*-NPP correlated with substrate

selectivity *in vitro* and *in vivo*.[24,31] Despite the fact that complexes between wild-type CL100 (MKP-1) and endogenous ERK2 could not be detected in Cos-1 cells[21] recombinant CL100 protein also undergoes catalytic activation. However, in contrast to Pyst1 and Pyst2 this enzyme is activated by recombinant ERK2 and both JNK1 and p38 SAP kinases (our unpublished observations). This reflects the ability of CL100 to dephosphorylate these different MAP and SAP kinases *in vivo* and indicates that catalytic activation is a general property of the MKPs. Presumably the physical interaction between CL100 and its substrates is less robust and specific than that between Pyst1 and ERK2 and escapes detection by co-immunoprecipitation.

What then is the physical basis for substrate binding and catalytic activation? Important clues have been provided by the recent determination of the crystal structure of the Pyst1 catalytic domain (Pyst1-CD).[32] The structure adopts a protein tyrosine phosphatase fold with a shallow active site. The latter feature is also found in the dual-specificity phosphatase VHR and has been suggested to account for the ability of these enzymes to accommodate both phosphotyrosine and phosphoserine/threonine within the active site cleft.[33] The most striking feature of the Pyst1-CD structure is that the active site displays a distorted geometry in the absence of substrate. Firstly, a highly conserved arginine residue (Arg 299 in Pyst1) which, in other PTPases, coordinates the phosphate group of the substrate, is not well positioned to assist in catalysis. Interestingly, a structure has recently been determined for the cdc25 cell cycle control phosphatase which functions by dephosphorylating adjacent threonine and tyrosine residues within $p34^{cdc2}$ [34] Cdc25 also exhibits an unconstrained side chain conformation for this active site arginine residue and it is interesting to speculate that this flexibility may be related to the ability of both cdc25 and Pyst1 to recognise diphosphorylated protein substrates.

Finally, by analogy with the VHR dual-specificity phosphatase, a highly conserved aspartic acid residue (Asp262 in Pyst1) is predicted to perform an essential role in catalysis by acting as a general acid responsible for protonation of the tyrosine leaving group.[35] Surprisingly, substitution of this residue by asparagine (D262N) in both full-length Pyst1 and Pyst1-CD has no effect on rate and specificity constants for the hydrolysis of *p*-NPP in the absence of ERK2, indicating that Asp 262 is not essential for activity.[32] This contrasts with results obtained for VHR and PTP1B where substitution of this residue decreases activity by several orders of magnitude.[36] However, the D262N mutant of Pyst1 fails to undergo catalytic activation towards *p*-NPP in the presence of ERK2, indicating that this residue is only critically required for the high activity form of Pyst1. This mutant is also severely compromised in its ability to dephosphorylate and inactivate ERK2 *in vivo* but is still able to co-associate with ERK2 (our unpublished observations). In the absence of ERK2 the position of Asp 262 in Pyst1-CD is displaced by almost 5.5 Å from the equivalent position in VHR. One explanation of these data is that Asp 262 and its associated loop undergo closure over the active site only upon binding of the phosphatase to ERK2, thus positioning the aspartate residue in a conformation in which it can participate in catalysis. However, the precise nature of the conformational rearrangement necessary for the catalytic activation of Pyst1 must await the structure of a co-complex of Pyst1 bound to MAP kinase.

Both Tyrosine-specific and Serine/Threonine Protein Phosphatases also Play a Role in Regulating MAP and SAP Kinase Signalling

The first evidence for the direct involvement of tyrosine-specific protein phosphatases in the regulation of MAP kinase activity came from studies of the osmoregulatory MAP kinase pathways in yeasts. In *S. cerevisiae* the Hog1p MAP

kinase, which is most highly related to the mammalian p38 SAP kinase, is activated in response to osmotic stress. Genetic and biochemical studies have revealed that this MAP kinase is inactivated by two tyrosine-specific phosphatases encoded by *PTP2* and *PTP3*.[37,38]

In *S. pombe*, the functional equivalent of this pathway contains the Sty/Spc MAP kinase which is also inactivated by two tyrosine-specific phosphatases *Pyp1*$^+$ and *Pyp2*$^+$.[39,40] Furthermore the gene encoding Pyp2 is transcriptionally upregulated in response to stress and this is mediated by the transcription factor Atf-1 which lies downstream of Sty/Spc. Thus, like the inducible dual-specificity MKPs, these enzymes may function in negative feedback loops to regulate MAP kinase activity. In a recent twist to this story Zhan *et al.* found that *PTP3*, in addition to its role in regulating Hog1p in *S. cerevisiae*, is also involved in regulating the pheromone responsive Fus3p MAP kinase, indicating that multiple classes of protein phosphatases may be involved in regulating a single MAP kinase.[41]

Given the degree to which MAP kinase signalling pathways are conserved from yeasts to man it would be surprising if mammalian equivalents of these tyrosine-specific MAP kinase phosphatases did not exist. Indeed biochemical studies both in mammalian cells[42] and in *Xenopus*[43] have detected such activities. Recent work by Pulido *et al.*, has now led to the characterisation of two enzymes which are expressed predominantly in neuronal cells and which may be the first members of a family of such proteins.[44] The PTPases PTP-SL and STEP are related proteins which exist in both transmembrane and cytosolic forms. In a search for physiological substrates for these PTPases, it was found that both PTP-SL and STEP associate with and are both substrates and inactivators of members of the MAP kinase family of enzymes. Like the dual-specificity MKPs these proteins form complexes with the ERK1/2 MAP kinases via an interaction motif located in their non-catalytic amino-terminal domain. However, this motif shows no sequence homology to the corresponding region of the dual-specificity MKPs. It remains to be seen if such proteins are more widely expressed in human cells and tissues and whether their activity is restricted to the mitogen rather than the stress-activated isoforms of MAP kinase.

A role for serine/threonine specific protein phosphatases in direct regulation of MAP kinase activity is more controversial. There is biochemical evidence which suggests a role for the serine/threonine phosphatase PP2A in regulating ERK2 activity in certain mammalian cell lines.[42] Studies in yeasts have suggested that *PTC1*, which encodes a serine/threonine protein phosphatase type-2C (PP2C), is a negative regulator of the Hog1p MAP kinase in *S. cerevisiae*.[37,45] A similar negative regulatory role was suggested for the *S. pombe* PP2C encoded by *ptc1*$^+$.[39,46] However, more recent studies in fission yeast have indicated that the target for this phosphatase is a downstream element of the Sty1/Spc1 MAP kinase pathway rather than the MAP kinase itself.[47] Nevertheless, Takekawa *et al.* performed a genetic screen in *S. cerevisiae* looking for mammalian cDNAs which were capable of downregulating the Hog1p MAP kinase and isolated human protein phosphatase 2Cα (PP2Cα).[48] They went on to demonstrate that this enzyme was able to inhibit the activation of both the p38 (SAPK2) and JNK1 SAP kinase cascades in mammalian cells. Interestingly, this occurred at both the level of the MAP kinase kinases MKK6 and SEK1 and the p38 MAP kinase itself and a direct interaction between PP2Cα and p38 was demonstrated by co-immunoprecipitation. No evidence for the regulation of this phosphatase by any of the stresses which activate these SAP kinase pathways was found, leading the authors to speculate that PP2Cα might be responsible for preventing spontaneous or inappropriate activation of these SAP kinase pathways. This may be important in achieving a low signal to noise ratio by maintaining pre-stimulation MAP kinase activity at a minimum.

CONCLUDING REMARKS

The past five years have seen an explosion of interest in the MAP kinase signalling pathways. This has encompassed studies of their mode of activation, cellular targets and physiological functions. Our knowledge about the role of protein phosphatases in regulating the activity of these key enzymes has also increased enormously over this period, with the discovery that multiple classes of protein phosphatases are involved in the regulated inactivation of these enzymes in mammalian cells. In particular we have considerable insight into the molecular mechanisms by which dual-specificity MAP kinase phosphatases are able to interact with and are activated by their target MAP and SAP kinase substrates. The challenge for the future is to gain an understanding of how these various enzymes are integrated to fine tune signalling through the different MAP and SAP kinase modules, what role these proteins might play in cross-regulation of one MAP kinase pathway by another and precisely how they influence the physiological outcome of MAP kinase signalling in mammalian cells and tissues.

Acknowledgements

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