Protein phosphatase 2C α inhibits the human stress-responsive p38 and JNK MAPK pathways

Mutsuhiro Takekawa, Tatsuya Maeda and Haruo Saito¹

Dana-Farber Cancer Institute and Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, MA 02115, USA

¹Corresponding author
e-mail: haruo_saito@dfci.harvard.edu

MAPK (mitogen-activated protein kinase) cascades are common eukaryotic signaling modules that consist of a MAPK, a MAPK kinase (MAPKK) and a MAPKK kinase (MAPKKK). Because phosphorylation is essential for the activation of both MAPKKs and MAPKs, protein phosphatases are likely to be important regulators of signaling through MAPK cascades. To identify protein phosphatases that negatively regulate the stress-responsive p38 and JNK MAPK cascades, we screened human cDNA libraries for genes that down-regulated the yeast HOG1 MAPK pathway, which shares similarities with the p38 and JNK pathways, using a hyperactivating yeast mutant. In this screen, the human protein phosphatase type 2C α (PP2C α) was found to negatively regulate the HOG1 pathway in yeast. Moreover, when expressed in mammalian cells, PP2C α inhibited the activation of the p38 and JNK cascades induced by environmental stresses. Both *in vivo* and *in vitro* observations indicated that PP2C α dephosphorylated and inactivated MAPKKs (MKK6 and SEK1) and a MAPK (p38) in the stress-responsive MAPK cascades. Furthermore, a direct interaction of PP2C α and p38 was demonstrated by a co-immunoprecipitation assay. This interaction was observed only when cells were stimulated with stresses or when a catalytically inactive PP2C α mutant was used, suggesting that only the phosphorylated form of p38 interacts with PP2C α .

Keywords: MAP kinase/protein kinase/protein phosphatase/signal transduction/stress response

Introduction

MAPK (mitogen-activated protein kinase) cascades are intracellular signaling modules composed of three tiers of sequentially activating protein kinases, which commonly are referred to as MAP kinase (MAPK), MAPK kinase (MAPKK) and MAPKK kinase (MAPKKK) (Marshall, 1994). An activated MAPKKK phosphorylates and activates a specific MAPKK, which then activates a specific MAPK. In mammalian cells, three distinct MAPK cascades have been identified. The prototypic MAPKs, ERK1/ERK2, are activated by mitogenic stimulation via RAF1 (MAPKKK) and MEK1/MEK2 (MAPKK) (Cobb and Goldsmith, 1995). In contrast, the two other types of

MAPKs, namely Jun-N-terminal kinase (JNK; also known as SAPK) and p38, are activated by environmental stresses such as osmotic shock, UV irradiation, heat shock, wound stress and inflammatory factors (Waskiewicz and Cooper, 1995; Kyriakis and Avruch, 1996; Ip and Davis, 1998). JNK1/JNK2 are activated by SEK1 or MKK7, whereas p38 is activated by either MKK3 or MKK6. Numerous candidates for MAPKKKs that activate SEK1, MKK3 and/or MKK6 have been reported, but their specific functions are unclear (Kyriakis and Avruch, 1996; Ip and Davis, 1998).

Yeast (*Saccharomyces cerevisiae*) also has several MAP kinase cascades that transduce distinct extracellular stimuli (e.g. high osmolarity) (Herskowitz, 1995; Posas *et al.*, 1998). Extracellular hyperosmolarity is detected by either of two transmembrane osmosensors, SHO1 and SLN1 (Maeda *et al.*, 1994, 1995). SLN1 is a homolog of prokaryotic two-component signal transducers, and transmits signals to the redundant SSK2 and SSK22 MAPKKKs, via the SLN1–YPD1–SSK1 multi-step phosphorelay system (Maeda *et al.*, 1994, 1995; Posas *et al.*, 1996; Posas and Saito, 1998). Activated SSK2 or SSK22 MAPKKK phosphorylates and activates the PBS2 MAPKK, which then activates the HOG1 MAPK (Brewster *et al.*, 1993; Maeda *et al.*, 1995).

The HOG1 MAPK cascade shares many structural and functional similarities with the mammalian counterparts. For example, the yeast PBS2 and mammalian MKK3/MKK6 MAPKKs are structurally very similar, as are the yeast HOG1 and mammalian p38 MAPKs (Boguslawski and Polazzi, 1987; Brewster *et al.*, 1993; Han *et al.*, 1994; Dérijard *et al.*, 1995). Furthermore, we recently identified a human MAPKKK termed MTK1, which is homologous to the yeast SSK2/SSK22 MAPKKKs (Takekawa *et al.*, 1997). Perhaps more important than their structural similarities, human p38 and MTK1 can functionally complement the osmoregulatory defects in yeast *hog1* and *ssk2* mutants, respectively (Han *et al.*, 1994; Takekawa *et al.*, 1997). The structural and functional similarities between the yeast HOG1 pathway and the mammalian p38 and JNK pathways suggest that they might also share similar regulatory mechanism(s).

Our previous studies indicated that two types of protein phosphatases, namely a protein tyrosine phosphatase (PTPase) and a serine/threonine protein phosphatase type 2C (PP2C), have important negative regulatory roles in the HOG1 osmoregulatory pathway. Initially, this was discovered through the following observations. Mutants of the SLN1 osmosensor are lethal because of inappropriate hyperactivation of the HOG1 MAPK (note that SLN1 is a negative regulator of the HOG1 pathway) (Maeda *et al.*, 1994; Posas *et al.*, 1996). This *sln1* lethality is suppressed if HOG1 MAPK activation is prevented, e.g. by disruption of the *PBS2* or *HOG1* gene (Maeda *et al.*, 1994). *sln1*

lethality can also be suppressed by overexpression of either of two protein phosphatases, PTP2 (a PTPase) or PTC1 (a PP2C) (Maeda *et al.*, 1994; Wurgler-Murphy *et al.*, 1997). The importance of the PTP2 and PTC1 phosphatases in the HOG1 pathway was also demonstrated by the synthetic lethality of the *ptp2 ptc1* double mutation, which causes hyperactivation of the HOG1 MAPK (Maeda *et al.*, 1994). Furthermore, the lethality of *ptp2 ptc1* double mutation can be suppressed by deletion of the *HOG1* gene (T.Maeda, S.Wurgler-Murphy and H.Saito, unpublished observation). Similar negative regulatory roles were proposed for PTPases and PP2C in the fission yeast (*Schizosaccharomyces pombe*) stress-induced MAPK cascade (Millar *et al.*, 1995; Shiozaki and Russell, 1995a,b; Gaits *et al.*, 1997).

Given the extent of conservation between yeast and mammalian MAPK pathways, it can be expected that a human regulatory molecule of p38 and/or JNK pathways also similarly regulates the yeast HOG1 pathway, when it is expressed in yeast. Thus, we undertook to screen a human cDNA library for clones which could block the activation of the yeast HOG1 MAPK pathway, in order to find negative regulators of the human p38 and/or JNK pathways. Here we demonstrate that a human protein phosphatase, PP2C α , inhibits the activation of the yeast HOG1 pathway and, more importantly, negatively regulates the human stress-responsive p38 and JNK MAPK pathways. *In vivo* and *in vitro* studies indicate that PP2C α dephosphorylates both MAPKK (MKK6 and SEK1) and a MAPK (p38). Thus, PP2C-type enzymes have a conserved function in negatively regulating stress-responsive MAPK cascades both in higher and lower eukaryotic cells.

Results

Identification of a human gene that negatively regulates the yeast HOG1 MAP kinase pathway

In order to identify human genes that negatively regulate the p38 and JNK stress-activated MAPK cascades, we devised a gene screening approach based on the following rationale. Previously, it was shown that the yeast HOG1 osmoregulatory MAP kinase cascade, which is composed of the SSK2/SSK22 MAPKKs, PBS2 MAPKK and HOG1 MAPK, is structurally similar to the mammalian stress-induced MAPK cascades. Moreover, the corresponding human genes partially complement the yeast mutants in the HOG1 pathway (Galcheva-Gargova *et al.*, 1994; Han *et al.*, 1994; Dérijard *et al.*, 1995; Takekawa *et al.*, 1997). Thus, it could be anticipated that human genes whose normal function is to repress the stress-inducible MAPK cascade also inhibit the yeast HOG1 pathway. As described in the Introduction, yeast *sln1* mutants are lethal because of the constitutive hyperactivation of the HOG1 MAPK cascade. Because the *sln1* lethality can be completely suppressed by inhibiting HOG1 activation, we reasoned that expression of mammalian genes that negatively regulate the HOG1 MAPK cascade would also suppress the *sln1* lethality.

Thus, we sought to isolate human genes that suppress the lethality of a *sln1^{ts}* mutant strain at the non-permissive temperature, by expressing human cDNAs made in a yeast expression vector. The spontaneous reversion frequencies of haploid *sln1* mutants are relatively high ($\sim 10^{-4}$), due

Fig. 1. Expression of human MC4 (PP2C α) inhibits the yeast HOG1 MAPK cascade. (A) Expression of the human MC4 cDNA suppresses the conditional lethality of the yeast *sln1^{ts}* mutant. TM251 (*sln1-ts4/sln1-ts4*) cells transformed with either pGAL vector or pGAL-MC4 were grown at 30 or 37°C on SC plates containing glucose or galactose. (B) Expression of the human MC4 cDNA suppresses the lethality of SSK2 Δ N, SSK22 Δ N and PBS2^{DD} mutations in yeast. The wild-type yeast strain TM141 transformed with either pGAL vector, pGAL-SSK2 Δ N, pGAL-SSK22 Δ N or pGAL-PBS2^{DD} in combination with either pTEF vector or pTEF-MC4 were grown at 30°C on SC plates containing glucose (Glc) or galactose (Gal).

to the frequent occurrence of extragenic suppressor mutations in one of the four downstream genes (*SSK1*, *SSK2*, *PBS2* and *HOG1*) (Maeda *et al.*, 1994). The reversion frequency could be significantly lowered, however, by using a diploid *sln1^{ts}/sln1^{ts}* strain. As an additional means to distinguish the suppression by expression of human cDNAs from endogenous suppressor mutations, we used a human cDNA library made in an expression vector (pNV7) that carries the inducible GAL1 promoter (*P_{GAL1}*). In this manner, suppression of *sln1^{ts}* by cloned human cDNA sequences should occur only on galactose-containing media but not on glucose-containing media, whereas the suppression of the *sln1* lethality by an endogenous suppressor mutation is independent of the sugar source.

The yeast strain TM251 (*sln1^{ts}/sln1^{ts}*) was transformed with a HeLa cell cDNA library made in pNV7, and $\sim 10^6$ transformants were screened for growth at the non-permissive temperature (37°C) on SC + galactose plates. Temperature-resistant transformants were then tested for their growth on SC + glucose plates at 37°C. Using this approach, we identified a human cDNA clone termed MC4 that reproducibly restored the TM251 viability at 37°C on galactose-containing media, but not on glucose-containing media (Figure 1A). In order to characterize further the effect of MC4 expression on the yeast HOG1 pathway, we utilized our previous observations that the HOG1 pathway can be activated by expressing either constitutively active MAPKKK mutants (SSK2 Δ N or SSK22 Δ N) or a constitutively active MAPKK mutant (PBS2^{DD}) (Wurgler-Murphy *et al.*, 1997). These mutant enzymes are active irrespective of the state of the upstream signaling elements. The hyperactivation of the HOG1 MAPK caused by any of these mutant kinases is lethal, apparently for the same reason as the lethality of the *sln1* mutants (Maeda *et al.*, 1995; Wurgler-Murphy *et al.*,

Fig. 2. MC4 encodes a human PP2C α splice variant. (A) Schematic diagram comparing PP2C α 1 and PP2C α 2 (MC4). The two forms differ only outside the conserved PP2C catalytic domain. (B) Sequence comparison between the C-termini of PP2C α 1 and PP2C α 2. Bold letters indicate the sequences specific for PP2C α 1 or PP2C α 2. Numbers in parentheses are the positions of the amino acids shown. *, stop codon. The total nucleotide sequence data of the MC4 cDNA clone has been submitted to the DDBJ/EMBL/GenBank databases under accession No. AF070670.

1997). As shown in Figure 1B, expression of MC4 suppressed the lethality caused by these constitutively active kinases, providing additional evidence that MC4 inhibits the activation of the HOG1 pathway. It can be also deduced from these data that the inhibitory effect of MC4 is targeted toward the MAPK cascade, rather than its upstream signaling elements such as the SLN1 histidine kinase or the SSK1 response regulator.

MC4 encodes a splice variant of human protein phosphatase 2 α (PP2C α)

Sequence determination of the 2.1 kb MC4 cDNA demonstrated that MC4 encoded a variant of human PP2C α (Figure 2A). Nucleotides 1–238 and 341–1311 of MC4 (accession No. AF070670) are 100% identical to nucleotides 101–338 and 339–1309, respectively, of the previously reported human PP2C α sequence (accession No. S87759; Mann *et al.*, 1992). An open reading frame (ORF) is found in MC4 between nucleotides 360 and 1334, which would encode a protein of 324 amino acids. The extra nucleotide sequence in MC4 near its 5' end (nucleotides 239–340) is totally within the 5'-untranslated region, and does not affect the structure of the encoded protein. In contrast, the divergence between the MC4 sequence and the human PP2C α sequence after nucleotide position 1311 leads to a corresponding divergence of their protein sequences. The N-terminal 317 amino acids were identical between the MC4 sequence and the previously reported human PP2C α sequence (Mann *et al.*, 1992). However, the MC4 sequence contained only seven amino acids in the C-terminus, whereas the PP2C α sequence contained 65 additional amino acids (Figure 2B). Because of the extensive identity between the MC4 and PP2C α nucleotide sequences, it is likely that they are derived from the same gene by alternative splicing. However, it will be necessary to determinate the PP2C α genomic structure in order to ascertain the mechanism of generating the two different PP2C α messages. Thus, we named the protein encoded by MC4 PP2C α 2; the original PP2C α will be called PP2C α 1. The conserved catalytic domain that is defined by the sequence similarity among known PP2C enzymes (Das *et al.*, 1996) is completely included within the common N-terminal segment of the two isoforms. The catalytic domain of PP2C α shares 35% sequence identity with yeast PTC1.

Fig. 3. Expression of PP2C α 1 and PP2C α 2 mRNA. A Northern blot (Clontech) containing poly(A)⁺ RNA isolated from various human tissues was hybridized with a probe specific to either PP2C α 1 (nucleotides 1349–2317; accession No. S87759) or PP2C α 2 (nucleotides 1312–2100; accession No. AF070670), as described previously (Takekawa *et al.*, 1997).

Northern blot analysis of mRNA from various human tissues using the unique 3'-non-coding sequences of the PP2C α 1 and PP2C α 2 isoforms as hybridization probes revealed a single 2.8 kb transcript for PP2C α 1 and a 4.4 kb transcript for PP2C α 2 (Figure 3). A probe that hybridizes to sequences common to PP2C α 1 and PP2C α 2 detected both transcripts, indicating that the PP2C α 2 isoform is not an artifact of cDNA cloning, such as ligation of two unrelated cDNA sequences (data not shown). In MRC5 (human lung fibroblast) cells, the ratio of the amounts of PP2C α 1 mRNA to PP2C α 2 mRNA was ~10:1. Both PP2C α 1 and PP2C α 2 mRNAs are expressed at high levels in heart, placenta, skeletal muscle and pancreas; PP2C α 2 is also expressed in the brain. Because we found no significant differences between PP2C α 1 and α 2 in the *in vivo* functional studies using human cells, only results obtained with PP2C α 2 will be presented in most cases. Further investigation might, however, reveal qualitative and/or quantitative differences between the two splice isoforms.

PP2C α inhibits p38 and JNK1 MAPK activation in human cells

Because the expression of human PP2C α 2 in yeast blocks the activation of the HOG1 MAPK pathway, it is possible that PP2C α has a similar role in mammalian cells. Therefore, we tested whether expression of PP2C α has any effect on the activation of the mammalian stress-responsive (p38 and JNK) MAPK cascades, as well as the mammalian mitogen-responsive (ERK) MAPK cascade.

Initially, COS-7 cells were transfected with expression plasmids encoding either PP2C α 1 or PP2C α 2, together with the hemagglutinin (HA)-tagged MAPK constructs, HA-p38, HA-JNK1 or HA-ERK2. Transfected cells were stimulated with either osmotic stress (sorbitol) or with mitogen phorbol-12-myristate-13-acetate (PMA). HA-tagged MAPKs were immunoprecipitated, and their kinase activities were determined *in vitro* using specific substrates (GST-ATF2 for p38, GST-Jun for JNK1 and MBP for ERK2). As shown in Figure 4 (left panels), both PP2C α 1 and PP2C α 2 suppressed the stress-induced activation of p38 and JNK1. In clear contrast, neither PP2C α 1 nor PP2C α 2 had any effect on the mitogen-induced activation of ERK2.

In order to obtain a more quantitative estimate of the

Fig. 4. Selective inhibition of the p38 and JNK MAPK pathways by PP2C α *in vivo*. COS-7 cells (left panel) were transfected with 0.7 μ g of expression plasmid encoding HA-p38, HA-JNK1 or HA-ERK2, together with 1.4 μ g of pcDNAI-Amp (control), pPP2C α 1 or pPP2C α 2. HeLa cells (right panel) were co-transfected with 0.5 μ g of individual MAPK expression plasmids and the indicated amounts of pPP2C α 2. Transfected cells were serum starved and then stimulated with 0.4 M sorbitol or 100 nM PMA for 20 min. The activities of epitope-tagged MAPKs were measured by an immunocomplex kinase assay using exogenous substrates (GST-ATF2, GST-Jun or MBP). The expression levels of MAPK in cell lysates were monitored by immunoblotting.

effect of PP2C α on the activation of the p38, JNK and ERK MAPKs, HeLa cells were transfected with various amounts of the PP2C α 2 expression plasmid DNA together with a constant amount of the tagged MAPK constructs. The total amount of transfected plasmid DNA was kept constant by including appropriate amounts of vector plasmid (pcDNAI-Amp). Activation of the p38 and JNK1 MAPKs induced by osmotic stress was suppressed in a dose-dependent manner by increasing amounts of PP2C α 2 expression plasmid (Figure 4, right panels). Expression of PP2C α 2 in HeLa cells had little inhibitory effect on the activation of the ERK2 MAPK by mitogenic stimulus, consistent with the COS-7 data. In summary, it is concluded that the expression of PP2C α strongly inhibits the activation of the stress-responsive MAPKs (p38 and JNK1), but not that of the mitogen-induced ERK2 MAPK.

PP2C α inhibits activation of the p38 and JNK1 MAP kinases induced either by environmental stresses or by pro-inflammatory cytokines

In addition to being activated by environmental stresses such as osmotic stress, UV and the ribosome inhibitor anisomycin, the p38 and JNK MAPK pathways can be activated by pro-inflammatory cytokines such as tumor necrosis factor- α (TNF- α). Although the same sets of MAPKKs and MAPKs are activated by these stimuli, the upstream signaling elements activated by environmental stresses appear to be different from those for the pro-inflammatory cytokines. For example, the small GTPases Rac and Cdc42 mediate the activation of p38 and JNK MAPKs by cytokines, but they are not involved in the activation of the same MAPKs by environmental stresses (Coso *et al.*, 1995; Minden *et al.*, 1995). In contrast, the

Fig. 5. PP2C α 2 inhibits the activation of the p38 and JNK pathways by distinct stimuli. HeLa cells were transfected with 1.5 μ g/plate of either pcDNAI-Amp or pPP2C α 2, together with Flag-p38 or HA-JNK1 expression plasmid (0.5 μ g). After 48 h, cells were treated with anisomycin (10 μ g/ml for 30 min), UV (80 J/m² followed by a 1 h incubation at 37°C), TNF- α (100 ng/ml for 15 min) or without any stimulation (-). The activities of Flag-p38 and HA-JNK1 were measured by an *in vitro* immunocomplex kinase assay.

MAPKKK MTK1 mediates the activation of p38 and JNK induced by environmental stresses, but not by cytokines (Takekawa *et al.*, 1997). Therefore, if PP2C α inhibits MAPK activation indirectly by interfering with some upstream elements, it will affect only one type of stimulus, but not others. On the other hand, if PP2C α inhibits either MAPKKs or MAPKs directly, PP2C α will inhibit activation of the p38 and JNK pathways irrespective of the inducing agent. As shown in Figure 5, PP2C α 2 suppressed the activation of p38 and JNK1 both by environmental stresses (anisomycin and UV) and by the cytokine TNF- α . Thus, it is likely that PP2C α regulates MAPKKs (MKK6 and SEK1) and/or MAPKs (p38 and JNKs).

PP2C α inactivates the stress-responsive MAPK pathways at the level of either MAPKK or MAPK

To determine the target(s) of PP2C α in the p38 and JNK MAPK pathways, we tested whether PP2C α can specifically inhibit MAPKK and/or MAPK *in vivo*. To test the effect of PP2C α expression on MAPKKs, COS-7 cells were co-transfected with a PP2C α 2 expression plasmid together with the GST-tagged MAPKK constructs, GST-MKK6, GST-SEK1 or GST-MEK1. After stimulation of the transfected cells with either osmotic stress (for MKK6 and SEK1) or PMA (for MEK1), the phosphorylation status of the GST-MAPKKs was probed with antibodies specific to the phosphorylated form of each MAPKK. The phosphorylation status of the MAPKK directly correlates with its catalytic activity (Raingeaud *et al.*, 1996). As shown in Figure 6A, PP2C α 2 reduced the level of MKK6 and SEK1 phosphorylation in a dose-dependent manner, whereas no detectable dephosphorylation of MEK1 was observed. These results suggest that PP2C α down-regulates the MKK6 and SEK1 MAPKKs *in vivo*, but not the mitogen-responsive MEK1.

We previously reported that overexpression of MTK1, a human homolog of the yeast SSK2/SSK22 MAPKKKs, leads to the activation of the MKK6 MAPKK (Takekawa *et al.*, 1997). To test if PP2C α inhibits the activation of MKK6 by MTK1, COS-7 cells were co-transfected with the MTK1 and MKK6 expression plasmids with or without the PP2C α expression plasmid. As seen in Figure 6B, co-expression of PP2C α almost completely inhibited MKK6

Fig. 6. Dephosphorylation and inactivation of stress-responsive kinases by PP2Cα *in vivo*. **(A)** Selective dephosphorylation of MKK6 and SEK1 MAPKKs by PP2Cα2 *in vivo*. COS-7 cells were transfected with 0.7 μg/plate of expression plasmid encoding GST-MKK6, GST-SEK1 or GST-MEK1, together with various amounts of pPP2Cα2. After 24 h, cells were serum starved for an additional 12 h, and then stimulated with 0.4 M sorbitol or 100 nM PMA for 20 min. The phosphorylation status of the affinity-purified GST-MAPKKs was analyzed by immunoblotting using antibodies specific to phosphorylated MKK6, SEK1 or MEK1. The expression level of each GST-MAPKK was also monitored using anti-GST antibody. **(B)** Inhibition of the MTK1-induced MKK6 phosphorylation by PP2Cα2 *in vivo*. COS-7 cells were transfected with GST-MKK6 (0.6 μg/plate), MTK1 (0.3 μg/plate) and PP2Cα2 (0.4 or 1.2 μg/plate) expression plasmids as indicated. After 36 h, serum-starved cells were lysed and the phosphorylation status of the affinity-purified GST-MKK6 was analyzed by immunoblotting using anti-phospho-specific MKK6 antibodies. The expression level of GST-MKK6 was monitored by anti-GST blot. **(C)** PP2Cα2 inhibits p38 activation induced by a constitutively active MKK6 mutant *in vivo*. HeLa cells were transfected with Flag-p38 (0.25 μg/plate), MKK6^{DD} (0.2 μg/plate) and PP2Cα2 (1.0 or 2.0 μg/plate) expression plasmids as indicated. After 24 h, the activity and expression level of Flag-p38 were determined as described in Figure 4.

activation by MTK1. This observation further strengthens our proposal that PP2Cα inhibits the activation of the stress-activated MAPKKs (e.g. MKK6 and SEK1), and possibly does so by directly dephosphorylating them.

While the above results suggest that PP2Cα acts on stress-responsive MAPKKs, another possibility, namely that PP2Cα could act on MAPKs, was suggested by the previous observations that expression of PP2Cα in yeast suppressed the lethal effect of the constitutively active PBS2^{DD} (Figure 1B). Because the activities of constitutively active MAPKK mutants, such as PBS2^{DD}, are independent of phosphorylation (Raingeaud *et al.*, 1996), one target of PP2Cα in yeast must be downstream of

PBS2 (it should be noted, however, that those experiments did not exclude the possibility that the PBS2 MAPKK was also a target of PP2Cα). In order to test if PP2Cα2 targets exist downstream of MKK6 or SEK1 MAPKKs, we did a similar epistasis test in mammalian cells using the constitutively active MKK6 allele, MKK6^{DD}. As seen in Figure 6C, PP2Cα2 expression strongly suppressed MKK6^{DD}-induced p38 activity. Because this suppression of p38 activation cannot be attributed to reduced MKK6^{DD} activity (its activity is independent of phosphorylation), it is concluded that PP2Cα can directly affect the activity of p38, most likely via dephosphorylation of activated p38. However, as noted for the similar epistasis test in yeast, this result does not exclude the possibility that PP2Cα also dephosphorylates MKK6 and SEK1 MAPKKs.

PP2Cα2 dephosphorylates the MKK6 and SEK1 MAPKKs, as well as the p38 MAPK *in vitro*

The *in vivo* experiments described above suggest that PP2Cα down-regulates the stress responses by inactivating MAPKKs and MAPKs in the p38 and JNK pathways. Whereas it was possible that PP2Cα directly dephosphorylated these kinases, it was also possible that the negative regulation was exerted indirectly by dephosphorylating another regulatory molecule. Thus, we tested if any, or all, of the putative targets of PP2Cα, namely MKK6 and SEK1 MAPKKs and p38 and JNK1 MAPKs, can serve as direct substrates for PP2Cα2 using an *in vitro* phosphatase assay. Initially, we tested whether purified GST-PP2Cα2 can dephosphorylate MAPKKs (GST-MKK6 and GST-SEK1) *in vitro*. GST-PP2Cα2 was expressed in *Escherichia coli* and purified as described in Materials and methods. In order to prepare phosphorylated MAPKKs, COS-7 cells were transfected with GST-MKK6 or GST-SEK1 expression plasmids and stimulated with osmotic stress. The phosphorylated GST-MKK6 or GST-SEK1 was affinity purified, and incubated with GST-PP2Cα2 *in vitro*. The phosphorylation status of GST-MAPKKs was then monitored by phospho-specific antibodies. As shown in Figure 7A, GST-PP2Cα2 dephosphorylated both GST-MKK6 and GST-SEK1 *in vitro*. In addition, dephosphorylation of GST-MKK6 and GST-SEK1 was dependent on the presence of Mg²⁺, which is consistent with the known properties of PP2C enzymes (Cohen, 1989).

We then tested whether PP2Cα2 can dephosphorylate MAPKs (p38 and JNK1) *in vitro*. This was done by measuring the effect of PP2Cα2 on MAPK activity of the MAPKs, because MAPK activity reflects their phosphorylation status. COS-7 cells were transfected with HA-p38 or HA-JNK1 expression plasmids, stimulated with osmotic stress, and activated HA-MAPKs were immunopurified. Their kinase activities were then measured *in vitro* using specific substrates (GST-ATF2 for p38 and GST-Jun for JNK1) before or after incubation with purified PP2Cα2. As seen in Figure 7A, PP2Cα2 efficiently inactivated p38 *in vitro*. In contrast, JNK1 activity was resistant to the action of the phosphatase. These results indicate that although PP2Cα2 can dephosphorylate several different substrates (MKK6, SEK1 and p38), it has an exquisite specificity. Namely, it can distinguish between two similar kinases, p38 and JNK1.

MAPK activity is dependent on phosphorylation at both

Fig. 7. Dephosphorylation of the MKK6, SEK1 and p38 by PP2C α 2 *in vitro*. (A) Activated MAPKKs or MAPKs were isolated from osmotic stress-treated COS-7 cells, incubated with various concentrations of purified GST-PP2C α 2 in the presence or absence of Mg²⁺, and subjected either to immunoblot analysis using phospho-specific antibodies (MKK6 and SEK1) or to an *in vitro* kinase assay (p38 and JNK1). (B) Threonine-specific dephosphorylation of ³²P-labeled p38 by PP2C α . MalE-p38 fusion protein was phosphorylated *in vitro* using purified GST-MKK6. Affinity-purified ³²P-labeled MalE-p38 was incubated with purified GST-PP2C α 2 in phosphatase buffer containing 30 mM MgCl₂ and 1 mg/ml BSA at 30°C for 20 min. Reaction products resolved by SDS-PAGE were subjected to phosphoamino acid analysis as described (Boyle *et al.*, 1991). The positions of the phosphoamino acids (P-Ser, P-Thr and P-Tyr) are indicated.

a threonine and a tyrosine residue (Anderson *et al.*, 1990; Gómez and Cohen, 1991). Because dephosphorylation of either site is sufficient to inactivate MAPKs, the inactivation of p38 by PP2C α can be mediated by dephosphorylation of either threonine or tyrosine residues. To test if PP2C α dephosphorylates the p38 MAPK at a threonine or a tyrosine phosphorylation site, ³²P-labeled p38 was dephosphorylated *in vitro* using purified PP2C α . For this purpose, a MalE-p38 fusion protein was expressed in *E. coli*, affinity purified and phosphorylated *in vitro* using GST-MKK6 and [γ -³²P]ATP. Phosphoamino acid analysis of ³²P-labeled MalE-p38 indicated, as expected, both threonine and tyrosine phosphorylation (Figure 7B). After incubation with GST-PP2C α 2, all the phosphate was removed from threonine, but not from tyrosine. Thus, it was concluded that PP2C α 2 dephosphorylates p38 at the essential threonine residue.

PP2C α binds the p38 MAP kinase *in vivo*

Finally, we tested if there is any direct interaction between PP2C α 2 and its substrate *in vivo*. For this purpose, Flag-tagged PP2C α 2 and HA-tagged p38 were co-expressed in COS-7 cells, and Flag-PP2C α 2 was immunoprecipitated before or after a brief osmotic stimulation. Immunoprecipitates were then probed with an anti-HA antibody to detect co-precipitated HA-p38. Interestingly, HA-p38 was associated with Flag-PP2C α 2 only after osmotic stimulation (Figure 8B). Because this observation suggested that PP2C α 2 preferentially binds phosphorylated p38, we tested if catalytically inactive PP2C α 2 bound p38 more efficiently. Similar trapping of specific substrates by catalytically inactive phosphatases has been observed in numerous studies (Sun *et al.*, 1993; Furukawa *et al.*, 1994; Brady-Kalnay *et al.*, 1995; Welihinda *et al.*, 1998). In order to generate a catalytically inactive PP2C α 2 mutant, we altered the conserved Arg195 to an Ala, because a mutational study with the *Caenorhabditis elegans* phos-

Fig. 8. Binding of PP2C α 2 to p38. (A) p38 MAPK activation is inhibited by wild-type PP2C α 2, but not by the catalytically defective PP2C α 2(R/A) mutant. HeLa cells were transfected with 0.5 μ g/plate of Flag-p38 plasmid, together with 1.5 μ g of expression plasmid for PP2C α 2, PP2C α 2(R/A) or their vector control (pcDNA1-Amp). After 48 h, transfected cells were treated with 0.4 M sorbitol for 20 min. p38 activity was measured by an *in vitro* kinase assay using GST-ATF2 as a substrate. The levels of Flag-p38 in total extracts are shown in the lower panel. (B) Co-immunoprecipitation of p38 and PP2C α 2. COS-7 cells were co-transfected with 1.0 μ g/plate of expression plasmid encoding HA-p38, together with 1.0 μ g of expression plasmid for Flag-tagged PP2C α 2 (wild-type or R/A mutant) or vector control (pcDNA1-Flag). Cells were harvested before (–) or 20 min after (+) treatment with 0.4 M sorbitol. Flag-PP2C α 2 was immunoprecipitated, and the presence of HA-p38 in the precipitates was detected by anti-HA antibody 12CA5 immunoblotting (upper panel). The levels of HA-p38 in total lysates are shown in the lower panel.

phatase FEM-2 suggested that a corresponding substitution mutation inactivates the phosphatase without major structural distortions (Chin-Sang and Spence, 1996). Indeed, purified mutant protein, PP2C α 2(R/A), did not dephosphorylate p38 *in vitro* (data not shown). Furthermore, expression of PP2C α 2(R/A) did not inhibit the activation of p38 *in vivo* under the conditions whereby expression of wild-type PP2C α 2 completely blocked p38 activation (Figure 8A). Furthermore, when Flag-PP2C α 2(R/A) was co-expressed with HA-p38, they were co-precipitated efficiently, whether the cells were stimulated with osmotic stress or not. Thus, it is likely that phosphorylated p38 is a high affinity substrate for mammalian PP2C α .

Discussion

Two types of protein phosphatases, serine/threonine-specific protein phosphatase type 2A (PP2A) and dual-specificity phosphatases, have been implicated in the negative regulation of mammalian MAPK cascades. PP2A dephosphorylates and inactivates ERK1/ERK2 MAPKs and MEK1/MEK2 MAPKKs (Gómez and Cohen, 1991; Frost *et al.*, 1994; Alessi *et al.*, 1995). However, it is unclear whether PP2A is similarly responsible for

dephosphorylation of stress-responsive MAPKs and MAPKKs, such as p38, JNK, MKK6 and SEK1. Dual-specificity phosphatases were shown to inactivate various members of the MAPK family by dephosphorylating both threonine and tyrosine residues (Charles *et al.*, 1993; Rohan *et al.*, 1993; Hunter, 1995; Keyse, 1995). Most dual-specificity phosphatases, including MKP-1, are highly inducible in response to mitogenic and/or stress stimuli, and are localized mainly in the nucleus (Charles *et al.*, 1993; Sun *et al.*, 1993), although another member of this family, MKP-3 (also known as VH-6 and PYST1), is constitutively expressed predominantly in the cytosol (Groom *et al.*, 1996; Muda *et al.*, 1996). These findings suggest that the major role of the dual-specificity enzymes is to inactivate MAPKs in the adaptation phase of a stimulation cycle.

Other studies, however, suggested that phosphatases other than PP2A and the dual-specificity phosphatases are required to down-regulate MAPK pathways, at least in some cell types (Alessi *et al.*, 1995). In this study, we demonstrated that the human PP2C α negatively regulates stress-responsive MAPK cascades. Specifically, PP2C α negatively regulates p38 and JNK stress-responsive MAPK pathways at two distinct levels. The p38 pathway is inhibited at both the MAPKK and MAPK levels, whereas the JNK pathway is inhibited only at the MAPKK level. The expression pattern of PP2C α suggests a specific role for PP2C α that is different from that of PP2A and the dual-specificity phosphatases in down-regulating stress-responsive MAPK pathways. PP2C α expression in mammalian cells is significantly different from that of dual-specificity phosphatases both temporally and spatially. Our Northern blot analysis indicated that expression of PP2C α is constitutive: the levels of both PP2C α 1 and α 2 mRNA in HeLa and MRC5 cells stayed nearly constant following high osmolarity shock (data not shown). Furthermore, experiments using green fluorescent protein (GFP)–PP2C α 1 and GFP–PP2C α 2 fusion proteins demonstrated that they were present in both the cytosol and the nucleus (data not shown), as previously reported for PP2C α 1 (Das *et al.*, 1996). These results thus suggest that PP2C α might be responsible for inactivation of the stress-induced MAPK cascades where most of the dual-specificity enzymes are not available. A likely scenario is that PP2C α serves to prevent spontaneous (or accidental) activation of the stress-responsive MAPK pathways when there is no stimulating signal. Such a mechanism is important to attain a high signal-to-noise ratio of MAPK activation by maintaining the pre-stimulation activity at its minimum.

Recently, an alfalfa PP2C termed MP2C was identified using inhibition of the yeast pheromone MAPK pathway as a basis for gene screening (Meskiene *et al.*, 1998). By epistasis analyses, it was found that expression of MP2C suppresses the lethality caused by a constitutively active STE11 MAPKKK mutant (*STE11-4*), but not the constitutively active SSK2 MAPKKK mutant (SSK2 Δ N) or the yeast PBS2 MAPKK mutant (PBS2^{DD}). Thus, MP2C appears to be specific to the enzymes in the pheromone-responsive MAPK pathway. In plants, MP2C may function as a negative regulator of the stress-activated MAPK (SAMK) pathway that is activated by cold, draught, touch and wounding (Meskiene *et al.*, 1998). The specific targets of MP2C have, however, not been identified. For that

matter, the specific targets of the yeast PP2C enzymes in stress-responsive MAPK pathways have remained controversial (Millar *et al.*, 1995; Shiozaki and Russell, 1995a,b; Gaits *et al.*, 1997). For example, Gait *et al.* (1997) suggested, based on genetic studies in *S. pombe*, that a PP2C enzyme acts either independently of, or downstream of, the MAP kinase cascade. Our current results, obtained by applying more direct biochemical approaches than the previous genetic approaches, provide strong support for the notion that PP2C inactivates stress-responsive MAPK pathways at the MAPKK and MAPK levels. Furthermore, these results indicate that negative regulation of MAPK cascades by PP2C enzymes is a common mechanism utilized by yeast, plant and mammalian cells.

Materials and methods

Yeast strains

The following yeast strains were used: TM141 (*MATa ura3 leu2 trp1 his3*) and TM251 (*MATa/MAT α sln1-ts4/sln1-ts4 ura3/ura3 leu2/leu2 TRP1/trp1 his3/HIS3*).

Media and buffers

Synthetic complete medium (SC) is supplemented with either 2% glucose (SD) or 2% galactose (SG). Lysis buffer is 20 mM Tris–HCl (pH 7.5), 1% Triton X-100, 10% glycerol, 137 mM NaCl, 2 mM EDTA, 50 mM β -glycerophosphate, 1 mM sodium vanadate, 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 μ g/ml leupeptin and 10 μ g/ml aprotinin. Phosphatase buffer is 50 mM Tris (pH 7.5), 0.1 mM EGTA and 0.1% 2-mercaptoethanol. Kinase buffer is 25 mM Tris–HCl (pH 7.5), 25 mM MgCl₂, 1 mM EGTA, 2 mM DTT, 0.5 mM sodium vanadate and 25 mM β -glycerophosphate. IP buffer is 50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 5 mM EGTA, 20 mM MgCl₂, 1 mM DTT, 1 mM PMSF, 10 μ g/ml leupeptin, 10 μ g/ml aprotinin, 50 mM NaF and 2 mM sodium vanadate. SDS loading buffer is 50 mM Tris–HCl (pH 6.8), 100 mM DTT, 2% SDS, 0.1% bromophenol blue and 10% glycerol.

Plasmids

Yeast expression plasmids pGAL-SSK2 Δ N, pGAL-SSK2 Δ N and pGAL-PBS2^{DD} have been described (Takekawa *et al.*, 1997; Wurgler-Murphy *et al.*, 1997). The full-length MC4 (PP2C α 2) cDNA was subcloned into the yeast expression vectors pYES2 (*P_{GAL}* promoter, *URA3*⁺ marker) and p423TEF (*P_{TEF}* promoter, *HIS3*⁺ marker) to construct pGAL-MC4 and pTEF-MC4, respectively. Mammalian expression plasmids pMT3-HA-p38, pSR α -HA-JNK1, pSR α -HA-ERK2, pCMV-Flag-p38, pEBG-MKK6, pEBG-MEK1 and pEBG-SEK1 were described (Takekawa *et al.*, 1997). The cDNAs encoding PP2C α 1 and PP2C α 2 were subcloned into the mammalian expression plasmid pcDNA1-Amp (Invitrogen) to generate pPP2C α 1 and pPP2C α 2. The phosphatase-deficient mutant PP2C α 2(R/A) was generated by replacing the Arg195 with an Ala codon using a PCR mutagenesis method (Maeda *et al.*, 1995). The constitutively active MKK6 mutant (MKK6^{DD}) was generated by replacing both Ser207 and Thr211 with Asp codons.

Isolation of *sln1* suppressor cDNA clones

The diploid *sln1*^{ts} mutant TM251 was transformed with HeLa and Jurkat cDNA libraries constructed in the yeast expression vector pNV7, in which human cDNA sequences are under the control of the inducible *P_{GAL}* promoter (Ninomiya-Tsuji *et al.*, 1991). Temperature-resistant transformants were selected on SG-Ura plates at 37°C. From ~10⁶ transformants, we identified three distinct plasmids: pMC2 (isolated twice), pMC4 (once) and pMC8 (isolated twice). Plasmid DNA was recovered from the transformed cells as described (Rose and Broach, 1991). pMC4 encoded a human PP2C α isoform. pMC2 and pMC8, which encoded unknown ORFs, were not studied in this work.

Northern blot analysis

The RNA tissue blot (Clontech) contains 2 μ g of poly(A)⁺ RNA isolated from various human tissues, fractionated by denaturing agarose gel and transferred onto a nylon membrane. The blot was hybridized, separately, to probes specific to either PP2C α 1 or PP2C α 2. Probe DNA was radioactively labeled by *E. coli* DNA polymerase (Klenow fragment) using random hexamers and [α -³²P]dCTP.

Tissue culture and transient transfection

COS-7 and HeLa cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, L-glutamine, penicillin and streptomycin. For transient transfection assays, cells grown in 35 mm dishes were transfected with the appropriate combinations of expression plasmids using lipofectamine (Gibco-BRL), according to the manufacturer's protocol. The total amount of plasmid DNA was adjusted to 2 μ g per plate with vector DNA (pcDNA1-Amp), and transfected cells were cultured overnight in serum-free medium. The cells were harvested 36 h after transfection and, where indicated, the cells were treated with sorbitol (400 mM for 20 min), anisomycin (10 μ g/ml for 30 min), UV-C (80 J/m² followed by a 1 h incubation at 37°C), TNF- α (100 ng/ml for 15 min) or PMA (100 nM for 20 min).

Immunoblotting

A 30 μ g aliquot of whole-cell lysates or appropriate amounts of glutathione-Sepharose affinity-purified proteins were resolved by SDS-PAGE and transferred onto nitrocellulose membranes. After blocking with 3.5% skim milk, membranes were probed with appropriate antibodies and visualized using enhanced chemiluminescence detection (Amersham). The following antibodies were used: mouse monoclonal antibody (mAb) 12CA5 specific to the HA epitope (Boehringer Mannheim); mouse mAb M2 specific to the Flag epitope (IBI-Kodak); and rabbit polyclonal antisera specific to phosphorylated SEK1, MKK6 and MEK1, respectively (New England Biolabs); and goat anti-GST antibody (Pharmacia).

Preparation of recombinant fusion proteins

GST fusion proteins GST-ATF2, GST-MKK6, GST-c-Jun and GST-PP2C α 2 were expressed in *E. coli* DH5 using the pGEX vector, and purified using glutathione-Sepharose beads as described previously (Takekawa *et al.*, 1997). MalE-p38 fusion protein was expressed in *E. coli* DH5 using the prokaryotic expression vector pMAL-cRI and purified using amylose resin according to the manufacturer's recommendations (New England Biolabs).

Immunoprecipitation and protein kinase assays

To assess the kinase activity of epitope-tagged MAPKs, transfected cells were lysed in the lysis buffer containing 0.5% deoxycholate. Cell lysates were incubated with the appropriate antibody for 2 h at 4°C. Immunocomplexes were recovered with the aid of Gamma-Bind-Sepharose beads (Pharmacia), washed twice with lysis buffer, once in 100 mM Tris-HCl (pH 7.5), 0.5 M LiCl, and twice with kinase buffer, as described previously (Coso *et al.*, 1995). Immunoprecipitates were resuspended in 30 μ l of the kinase buffer containing 3 μ g of specific substrate (GST-c-Jun for JNK1, GST-ATF2 for p38 or MBP for ERK2). The kinase reaction was initiated by addition of 10 μ Ci of [γ -³²P]ATP and 50 μ M cold ATP, as previously described (Minden *et al.*, 1995). After 30 min incubation at 30°C, reactions were terminated by adding 15 μ l of 3 $\times$ SDS loading buffer. Samples were boiled for 5 min, separated by SDS-PAGE on 12.5% polyacrylamide gels, dried and visualized by autoradiography.

In vitro phosphatase reactions

To prepare phosphorylated GST-tagged MAPKKs and HA-tagged MAPKs, COS-7 cells were transfected with plasmid DNA encoding either GST-MAPKK or HA-MAPK (pEBG-MKK6, pEBG-SEK1, pMT3-HA-p38 and pSR α -HA-JNK1), as described previously (Takekawa *et al.*, 1997). After stimulating cells with 0.4 M sorbitol, the activated (and therefore phosphorylated) MAPKKs and MAPKs were purified and used for *in vitro* phosphatase reactions as follows. Phosphorylated GST-MAPKKs were immobilized on glutathione-Sepharose, washed twice with lysis buffer, once with LiCl buffer (Takekawa *et al.*, 1997) and twice with phosphatase buffer, and divided into five aliquots. Each aliquot was resuspended in 50 μ l of phosphatase buffer supplemented with 1 mg/ml bovine serum albumin (BSA) with or without 30 mM MgCl₂. After addition of purified GST-PP2C α 2, reaction mixtures were incubated at 30°C for 15 min. Phosphatase reactions were terminated by adding 50 mM NaF, and resolved by SDS-PAGE. The phosphorylation status of MAPKKs was monitored by phospho-specific antibodies. Phosphorylated HA-MAPKs were immunopurified using anti-HA monoclonal antibody 12CA5 (Boehringer Mannheim). After treatment with PP2C α 2 in phosphatase buffer as described above, samples were washed three times with lysis buffer, twice with kinase buffer, and resuspended in 30 μ l of the kinase buffer. The remaining HA-MAPK activities were assayed by *in vitro* kinase reactions as described (Takekawa *et al.*, 1997).

Co-immunoprecipitation assay

Cell lysates were prepared in IP buffer containing 0.5% NP-40. A 600 μ g aliquot of cell extract was incubated with 3 μ g of anti-Flag mAb M2 (IBI-Kodak) for 6 h at 4°C, mixed with 35 μ l of Gamma-Bind Plus Sepharose suspension, and incubated for an additional hour. Immunoprecipitates were collected by centrifugation, washed six times with IP buffer plus 0.1% NP-40, and subjected to SDS-PAGE. Immunoblot analysis was performed with the anti-HA mAb 12CA5.

Acknowledgements

We thank E.A. Witten and M.T. Chen for their excellent technical assistance, A. Yamakawa for a plasmid, F. Posas and S. Wurgler-Murphy for suggestions, and M. Streuli, F. Posas, P.O. Grady and D. Raitt for valuable comments on the manuscript. This work was supported by NIH grants (GM50909 and GM56699) and a grant from Novartis/DFCI Drug Discovery Program to H.S., and a postdoctoral fellowship from the Human Frontier Science Program to M.T.

References

- Alessi, D.R., Gómez, N., Moorhead, G., Lewis, T., Keyse, S.M. and Cohen, P. (1995) Inactivation of p42 MAP kinase by protein phosphatase 2A and a protein tyrosine phosphatase, but not CL100 in various cell lines. *Curr. Biol.*, **5**, 283–295.
- Anderson, N.G., Maller, J.L., Tonks, N.K. and Sturgill, T.W. (1990) Requirement for integration of signals from two distinct phosphorylation pathways for activation of MAP kinase. *Nature*, **343**, 651–653.
- Boguslawski, G. and Polazzi, J.O. (1987) Complete nucleotide sequence of a gene conferring polymyxin B resistance on yeast: similarity of the predicted polypeptide to protein kinases. *Proc. Natl Acad. Sci. USA*, **84**, 5848–5852.
- Boyle, W.J., van der Geer, P. and Hunter, T. (1991) Phosphopeptide mapping and phosphoamino acid analysis by two-dimensional separation on thin-layer cellulose plate. *Methods Enzymol.*, **201**, 110–152.
- Brady-Kalnay, S.M., Rimm, D.L. and Tonks, N.K. (1995) Receptor protein tyrosine phosphatase PTP μ associates with cadherins and catenins *in vivo*. *J. Cell Biol.*, **130**, 977–986.
- Brewster, J.L., de Valoir, T., Dwyer, N.D., Winter, E. and Gustin, M.C. (1993) An osmosensing signal transduction pathway in yeast. *Science*, **259**, 1760–1763.
- Charles, C.H., Sun, H., Lau, L.F. and Tonks, N.K. (1993) The growth factor-inducible immediate-early gene *3CH134* encodes a protein-tyrosine-phosphatase. *Proc. Natl Acad. Sci. USA*, **90**, 5292–5296.
- Chin-Sang, I.D. and Spence, A.M. (1996) *Caenorhabditis elegans* sex-determining protein FEM-2 is a protein phosphatase that promotes male development and interacts directly with FEM-3. *Genes Dev.*, **10**, 2314–2325.
- Cobb, M.H. and Goldsmith, E.J. (1995) How MAP kinases are regulated. *J. Biol. Chem.*, **270**, 14843–14846.
- Cohen, P. (1989) The structure and regulation of protein phosphatases. *Annu. Rev. Biochem.*, **58**, 453–508.
- Coso, O.A., Chiariello, M., Yu, J.-C., Teramoto, H., Crespo, P., Xu, N., Miki, T. and Gutkind, J.S. (1995) The small GTP-binding proteins Rac1 and Cdc42 regulate the activity of the JNK/SAPK signaling pathway. *Cell*, **81**, 1137–1146.
- Das, A.K., Helps, N.R., Cohen, P.T. and Barford, D. (1996) Crystal structure of the protein serine/threonine phosphatase 2C at 2.0 Å resolution. *EMBO J.*, **15**, 6798–6809.
- Dérjard, B., Raingeaud, J., Barrett, T., Wu, I.-H., Han, J., Ulevitch, R.J. and Davis, R.J. (1995) Independent human MAP kinase signal transduction pathways defined by MEK and MKK isoforms. *Science*, **267**, 682–685.
- Frost, J.A., Alberts, A.S., Sontag, E., Guan, K., Mumby, M.C. and Feramisco, J.R. (1994) Simian virus 40 small t antigen cooperates with mitogen-activated kinases to stimulate AP-1 activity. *Mol. Cell Biol.*, **14**, 6244–6252.
- Furukawa, T., Itoh, M., Krueger, N.X., Streuli, M. and Saito, H. (1994) Specific interaction of the CD45 protein-tyrosine phosphatase with tyrosine-phosphorylated CD3 ζ chain. *Proc. Natl Acad. Sci. USA*, **91**, 10928–10932.
- Gaits, F., Shiozaki, K. and Russell, P. (1997) Protein phosphatase 2C acts independent of stress-activated kinase cascade to regulate the stress response in fission yeast. *J. Biol. Chem.*, **272**, 17873–17879.

- Galcheva-Gargova,Z., Dériard,B., Wu,I.-H. and Davis,R.J. (1994) An osmosensing signal transduction pathway in mammalian cells. *Science*, **265**, 806–808.
- Gómez,N. and Cohen,P. (1991) Dissection of the protein kinase cascade by which nerve growth factor activates MAP kinases. *Nature*, **353**, 170–173.
- Groom,L.A., Sneddon,A.A., Alessi,D.R., Dowd,S. and Keyse,S.M. (1996) Differential regulation of the MAP, SAP and RK/p38 kinases by Pyst1, a novel cytosolic dual-specificity phosphatase. *EMBO J.*, **15**, 3621–3632.
- Han,J., Lee,J.-D., Bibbs,L. and Ulevitch,R.J. (1994) A MAP kinase targeted by endotoxin and hyperosmolarity in mammalian cells. *Science*, **265**, 808–811.
- Herskowitz,I. (1995) MAP kinase pathways in yeast: for mating and more. *Cell*, **80**, 187–197.
- Hunter,T. (1995) Protein kinases and phosphatases: the yin and yang of protein phosphorylation and signaling. *Cell*, **80**, 225–236.
- Ip,Y.T. and Davis,R.J. (1998) Signal transduction by the c-Jun N-terminal kinase (JNK)—from inflammation to development. *Curr. Opin. Cell Biol.*, **10**, 205–219.
- Keyse,S.M. (1995) An emerging family of dual specificity MAP kinase phosphatases. *Biochim. Biophys. Acta*, **1265**, 152–160.
- Kyriakis,J.M. and Avruch,J. (1996) Sounding the alarm: protein kinase cascades activated by stress and inflammation. *J. Biol. Chem.*, **271**, 24313–24316.
- Maeda,T., Wurgler-Murphy,S.M. and Saito,H. (1994) A two-component system that regulates an osmosensing MAP kinase cascade in yeast. *Nature*, **369**, 242–245.
- Maeda,T., Takekawa,M. and Saito,H. (1995) Activation of yeast PBS2 MAPKK by MAPKKs or by binding of an SH3-containing osmosensor. *Science*, **269**, 554–558.
- Mann,D.J., Campbell,D.G., McGowan,C.H. and Cohen,P.T.W. (1992) Mammalian protein serine/threonine phosphatase 2C: cDNA cloning and comparative analysis of amino acid sequences. *Biochim. Biophys. Acta*, **1130**, 100–104.
- Marshall,C.J. (1994) MAP kinase kinase kinase, MAP kinase kinase and MAP kinase. *Curr. Opin. Genet. Dev.*, **4**, 82–89.
- Meskiene,I., Bögre,L., Glaser,W., Balog,J., Brandstötter,M., Zwerger,K., Ammerer,G. and Hirt,H. (1998) MP2C, a plant protein phosphatase 2C, functions as a negative regulator of mitogen-activated protein kinase pathways in yeast and plants. *Proc. Natl Acad. Sci. USA*, **95**, 1938–1943.
- Millar,J.B.A., Buck,V. and Wilkinson,M.G. (1995) Pyp1 and Pyp2 PTPases dephosphorylate an osmosensing MAP kinase controlling cell size at division in fission yeast. *Genes Dev.*, **9**, 2117–2130.
- Minden,A., Lin,A., Claret,F.-X., Abo,A. and Karin,M. (1995) Selective activation of the JNK signaling cascade and c-jun transcriptional activity by the small GTPases Rac and Cdc42Hs. *Cell*, **81**, 1147–1157.
- Muda,M., Theodosiou,A., Rodrigues,N., Boschert,U., Camps,M., Gillieron,C., Davies,K., Ashworth,A. and Arkinstall,S. (1996) The dual specificity phosphatases M3/6 and MKP-3 are highly selective for inactivation of distinct mitogen-activated protein kinases. *J. Biol. Chem.*, **271**, 27205–27208.
- Ninomiya-Tsujii,J., Nomoto,S., Yasuda,H., Reed,S.I. and Matsumoto,K. (1991) Cloning of a human cDNA encoding a CDC2-related kinase by complementation of a budding yeast *cdc28* mutation. *Proc. Natl Acad. Sci. USA*, **88**, 9006–9010.
- Posas,F. and Saito,H. (1998) Activation of the yeast SSK2 MAP kinase kinase by the SSK1 two-component response regulator. *EMBO J.*, **17**, 1385–1394.
- Posas,F., Wurgler-Murphy,S.M., Maeda,T., Witten,E.A., Thai,T.C. and Saito,H. (1996) Yeast HOG1 MAP kinase cascade is regulated by a multistep phosphorelay mechanism in the SLN1-YPD1-SSK1 ‘two-component’ osmosensor. *Cell*, **86**, 865–875.
- Posas,F., Takekawa,M. and Saito,H. (1998) Signal transduction by MAP kinase cascades in budding yeast. *Curr. Opin. Microbiol.*, **1**, 175–182.
- Raingeaud,J., Whitmarsh,A.J., Barrett,T., Dériard,B. and Davis,R.J. (1996) MKK3- and MKK6-regulated gene expression is mediated by the p38 mitogen-activated protein kinase signal transduction pathway. *Mol. Cell. Biol.*, **16**, 1247–1255.
- Rohan,P.J., Davis,P., Moskaluk,C.A., Kearns,M., Kruttsch,H., Siebenlist,U. and Kelly,K. (1993) PAC-1: a mitogen-induced nuclear protein tyrosine phosphatase. *Science*, **259**, 1763–1766.
- Rose,M.D. and Broach,J.R. (1991) Cloning genes by complementation in yeast. *Methods Enzymol.*, **194**, 195–230.
- Shiozaki,K. and Russell,P. (1995a) Cell-cycle control linked to extracellular environment by MAP kinase pathway in fission yeast. *Nature*, **378**, 739–743.
- Shiozaki,K. and Russell,P. (1995b) Counteractive roles of protein phosphatase 2C (PP2C) and a MAP kinase kinase homolog in the osmoregulation of fission yeast. *EMBO J.*, **14**, 492–502.
- Sun,H., Charles,C.H., Lau,L.F. and Tonks,N.K. (1993) MKP-1 (3CH134), an immediate early gene product, is a dual specificity phosphatase that dephosphorylates MAP kinase *in vivo*. *Cell*, **75**, 487–493.
- Takekawa,M., Posas,F. and Saito,H. (1997) A human homolog of the yeast Ssk2/Ssk22 MAP kinase kinase kinases, MTK1, mediates stress-induced activation of the p38 and JNK pathways. *EMBO J.*, **16**, 4973–4982.
- Waskiewicz,A.J. and Cooper,J.A. (1995) Mitogen and stress response pathways: MAP kinase cascades and phosphatase regulation in mammals and yeast. *Curr. Opin. Cell Biol.*, **7**, 798–805.
- Welihinda,A.A., Tirasophon,W., Green,S.R. and Kaufman,R.J. (1998) Protein serine/threonine phosphatase Ptc2p negatively regulates the unfolded-protein response by dephosphorylating Ire1p kinase. *Mol. Cell. Biol.*, **18**, 1967–1977.
- Wurgler-Murphy,S.M., Maeda,T., Witten,E.A. and Saito,H. (1997) Regulation of the *Saccharomyces cerevisiae* HOG1 mitogen-activated protein kinase by the PTP2 and PTP3 protein tyrosine phosphatases. *Mol. Cell. Biol.*, **17**, 1289–1297.

Received May 18, 1998; revised June 15, 1998;
accepted June 22, 1998