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Reconstitution of Novel Signalling Cascades Responding to Cellular Stresses [and Discussion]

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Reconstitution of novel signalling cascades responding to cellular stresses

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SUMMARY

Mammalian cells respond to their immediate environment by inducing signal transduction cascades that regulate metabolism, secretion and gene expression. Several of these signalling pathways are structurally and organizationally related insofar as they require activation of a protein-serine kinase via it's phosphorylation on tyrosine and threonine; the archetype being mitogen-activated protein kinase (MAPK) which responds primarily to mitogenic stimuli via Ras. In contrast, two more recently identified cascades are responsive to cellular stresses such as heat, inflammatory cytokines, ischaemia and metabolic poisons. The recent identification of the components of these pathways has allowed manipulation of the stress-responsive pathways and evaluation of their physiological roles. These studies reveal a high degree of independence between the pathways not apparent from *in vitro* studies. Manipulation of the pathways *in vivo* will likely result in novel therapies for inflammatory disease and reperfusion injury.

1. INTRODUCTION

Cells respond to a wide variety of specific cues such as growth factors, hormones, and cytokines, but also to environmental stresses such as thermal shock, irradiation, toxins and hypoxia. The mechanisms via which polypeptide growth factors elicit their pleiotropic effects have been the subject of intense study which has led to the elucidation of several transduction pathways and their components. Thus, binding of growth factors to their specific receptor tyrosine kinases induces dimerization and tyrosine autophosphorylation which recruits SH2 domain-containing proteins. One of these, Grb-2, is associated with the mSOS exchange factor which is translocated, as a consequence of Grb-2/receptor binding, to the membrane allowing interaction with it's target, Ras. The resultant stimulation of GTP binding to Ras recruits the Raf serine kinase which is also activated following movement to the membrane. Raf activates another protein kinase, MEK. This protein kinase targets a threonine and tyrosine residue on mitogen-activated protein kinases such as Erk1 and Erk2 (reviewed in Avruch *et al.* 1994).

The importance of this pathway is underscored by it's evolutionary conservation. In yeast, the response to mating factor acts through a series of gene products related to the MEK (STE7) and MAPK (FUS3, KSS1) enzymes (Neiman 1993). In *Drosophila*, the Torso receptor-tyrosine kinase has been genetically linked to D-Raf and Rolled (an Erk2 homologue; Hou *et al.* 1995). Furthermore, in mammalian cells the pathway contains two proto-oncogenes (Ras and Raf) and expression of a constitutively activated MEK protein results in transformation of fibroblasts indicating that activation of MAPKs is sufficient for abrogation of growth control (Cowley *et al.* 1994).

The mating factor pathway is not the only MAPK system in budding yeast. Genetic analysis has revealed at least three distinct pathways that are composed of three interacting protein kinases (analogous to MAPK, MEK and Raf). Aside from the mating factor response, yeast initiate signalling cascades in response to osmotic shock and factors affecting cell wall integrity (figure 1; reviewed in Herskowitz 1995). In the past three years, the existence of multiple 'three kinase' cascades has been demonstrated in higher eukaryotes including *Xenopus* and mammals; for review see Cano & Mahadevan (1995). Here, we describe the identification of the components of these new pathways and discuss their interrelations and physiological roles.

2. THE STRESS-ACTIVATED PROTEIN KINASE PATHWAY

Within minutes of cell stimulation by growth factors, programmes of gene transcription are altered within the nucleus (Hill & Treisman 1995). Because these events are largely independent of on-going protein synthesis, the response must involve changes in pre-existing transcription factors. One factor that has been extensively studied by virtue of it being the target of

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three independent retroviral transductions is AP-1. This factor comprises homo- and hetero-dimers of the Jun transcription factor family as well as hetero-dimers between Jun members and the Fos family. The complex is activated in response to a wide variety of stimuli including agents acting through Ras (AP-1 is required for Ras transformation; Lloyd *et al.* 1991).

Most of the component proteins of AP-1 are early inducible genes themselves. However, AP-1 activity can be induced in the presence of cycloheximide. The proteins responsible for this activity appear to be c-Jun and/or JunD depending on the cell type examined. These Jun proteins are phosphorylated in cells at several serine and threonine residues (Boyle *et al.* 1991). Three residues proximal to the DNA binding domain are phosphorylated in resting cells. Upon cell stimulation, these residues are dephosphorylated (Nikolakaki *et al.* 1993). Because phosphorylation of this region interferes with association of the Jun protein with DNA, this modification acts to repress the function of the transcription factor under resting conditions.

In addition to dephosphorylation of the residues near the DNA binding domain, many cell stimulants induce phosphorylation of two serine residues (serine 63 and 73) near the amino terminal transactivation domain (Pulverer *et al.* 1991; Smeal *et al.* 1991). Phosphorylation of these sites greatly enhances the transactivation potential of the Jun proteins, thus allowing efficient stimulation of the RNA polymerase II machinery (Franklin *et al.* 1993). Serines 63 and 73 are proceeded by proline residues which implicated a member of the MAPK family of enzymes in their regulation. Screening of MAPK preparations revealed these residues to be specifically targeted by a protein kinase termed p54 MAPK which had been purified to homogeneity from the livers of rats injected with cycloheximide (Kyriakis & Avruch 1990; Pulverer *et al.* 1991). This protein kinase had several properties associated with MAPKs including a Ser/Thr-Pro specificity and dependence on Ser/Thr and Tyr phosphorylation (Kyriakis *et al.* 1991).

cDNA clones of the p54 MAPK were isolated following determination of tryptic peptide sequences of the purified protein. These studies revealed the existence of three distinct genes encoding this protein kinase which were also subject to differential splicing to generate up to eight proteins of 54 kDa and 46 kDa. The three genes (termed α, β and γ) share 85–92% identity and all contain the sequence Thr-Pro-Tyr within a region analogous to the MAPK activation sites (Kyriakis *et al.* 1994). Overall identity to other MAPKs varies between 42–47% in the catalytic domain.

Polyclonal antibodies that recognized all of the protein kinase isoforms were generated and used to immunoprecipitate these kinases from a variety of cell types. Surprisingly, growth factors and mitogens acting through Ras only poorly activated these kinases (1.5–3 fold) compared with *bona fide* MAPKs (20–30 fold). In contrast, a number of other agents potently stimulated activity of the novel kinases while relatively poorly (if at all) activating the MAPKs (Kyriakis *et al.* 1994). These agents included tumour necrosis factor-α (TNF-α), IL-1, muscarinic acetylcholine receptors, anisomycin, cycloheximide, heat shock, ultra-violet light (UV), ischaemia–reperfusion and sodium arsenite (Kyriakis *et al.* 1994; Dérijard *et al.* 1994; Bird *et al.* 1994; Coso *et al.* 1994; Pombo *et al.* 1994; see table 1). In view of the generally stressful nature of these stimulants the novel kinases were termed Stress-Activated Protein Kinases (SAPKs).

Table 1. *Activation of SAPK and MAPK pathways by various agents*

(Assays were performed using GST-c-Jun as substrate for the SAPKs and myelin basic protein for MAPKs. SAPK activity was assayed following immunoprecipitation whereas MAPKs were resolved by Mono Q.)

agonist/mU	SAPK activity /mU	MAPK activity
control	7.5	65
anisomycin	520	120
UV light	375	270
heat shock (42 °C)	210	190
TNF-α	425	170
sodium arsenite	470	290
sphingomyelinase	190	ND
insulin	10.5	460
PDGF	9	480
carbacol	190	510
PMA	15	760

Members of the SAPK family have been independently cloned from other mammals. Whereas the α and γ genes are widely expressed SAPKβ expression is largely limited to nervous tissue, brain in particular (Mohit *et al.* 1995).

The differential activation of the MAPK and SAPK pathways by a wide variety of agents strongly suggested that these pathways shared few, if any, common components. In support of this, the MAPK kinase, MEK, does not phosphorylate SAPK *in vitro* or following transfection. Likewise, activated Raf and activated Ras do not immediately induce activation of the SAPKs. Use of an inducible Raf/oestrogen receptor fusion protein which allows rapid activation of Raf demonstrated that addition of oestrogen was accompanied with 30 min by MEK and MAPK activation. Stimulation of SAPK was observed following oestrogen addition, but with a delay of 8–24 h suggesting a requirement for secretion of a factor induced by MAPK activation (Minden *et al.* 1994).

Overall, the various SAPK gene products have quite similar properties including substrate specificity and activation (see below). In addition to c-Jun, the kinases phosphorylate several other transcription factors including ATF2, JunD and Elk1 (Gupta *et al.* 1995; J. R. Woodgett, unpublished observations). In the case of c-Jun and ATF2, the SAPKs display very high affinity and can be isolated from lysates complexed to these substrates (Dai *et al.* 1995; Livingstone *et al.* 1995; Van Dam *et al.* 1995). In the case of c-Jun, the presence of Ser 63/73 is not required for binding. Rather a hydrophobic domain of 20 amino acids is necessary and sufficient for the kinase–substrate interaction (Adler *et al.* 1994). Mutations in this region block

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binding and hence phosphorylation of c-Jun. This binding site corresponds to a region called the δ domain which is deleted in the virally transduced v-Jun oncoprotein suggesting it may contribute to this transforming proteins function. Indeed, v-Jun, although having the SAPK phosphorylation sites intact, is not a substrate for the kinase. However, v-Jun-mediated transactivation is independent of phosphorylation (Black *et al.* 1994). These observations raise the possibility that inactive SAPK bound to c-Jun represses the activity of this transcription factor in resting cells. Phosphorylation of Ser63/73 upon stimulation releases the kinase from the transactivation domain allowing productive interaction with the transcriptional machinery (Dai *et al.* 1995).

Of note, the SAPKα gene is differentially spliced between subdomains IX and X of the catalytic domain (Kyriakis *et al.* 1994). The two SAPKα proteins have very similar properties to each other and the other SAPKs but the αII gene exhibits a ~ 5-fold increased affinity for the c-Jun δ domain compared with its alternatively spliced cognate, SAPKαI (Dai *et al.* 1995). The significance of this difference in Jun regulation and its relation to other substrates is the subject of on-going studies.

(*a*) *SAPK regulation*

By analogy with the MAPKs, SAPKs are expected to be regulated by a protein kinase(s) specific for tyrosine and threonine residues, like MEK. Screening novel MEK-like proteins for phosphorylation of a kinase-dead SAPK mutant revealed a mouse homologue of a MEK-like kinase first identified in *Xenopus* (XMEK2; Yashar *et al.* 1993). This protein kinase, termed SEK1 (SAPK and Erk activating kinase 1), is widely expressed and specifically phosphorylates SAPK *in vitro* (Sanchez *et al.* 1994: also cloned as MKK4; Dérijard *et al.* 1995). Furthermore, SEK can be co-precipitated with SAPK when co-expressed in cells, regardless of the activation state. SEK1 is activated by the same agonists as SAPK and when transfected into cells is able to potentiate SAPK activation by agonists (Sanchez *et al.* 1994). Furthermore, transdominant SEK1 mutants effectively block SAPK activation in response to exogenous agonists. SEK1 is unlikely to be the only SAPK kinase as there is biochemical evidence suggesting SEK1 represents only a fraction of SAPK kinase activity in cell extracts (Moriguchi *et al.* 1995). The transdominant mutant SEK1 presumably exerts it's effect by binding to SAPKs thus interfering with their interaction with other SEK-like kinases.

The basis for the substrate specificity of SEK and MEK for SAPKs and MAPKs respectively, is likely related to the primary amino acid sequence surrounding the phosphorylated tyrosine and threonine residues. Thus, MAPKs all contain the sequence Thr-Glu-Tyr. The SAPKs, by contrast, have a proline residue between the phosphoacceptors. To date, the presence of TEY or TPY within the activation site is an accurate predictor of phosphorylation by MEK or SEK (see below and figure 1).

Although SEK has a low overall the amino acid identity to MEK, the two proteins are related in the region between kinase subdomains VII and VIII. This is the region harbouring the phosphorylation sites on MEK that are targeted by Raf. Alignment of the SEK and MEK sequences in this region reveals SEK Ser220 and Thr224 to be coincident with the two Raf targeted Ser residues in MEK (see figure 2). Mutation of these two residues to Ala and Leu respectively generates a transdominant mutant when expressed in Cos cells (Sanchez *et al.* 1994). These data strongly imply the existence of a SEK kinase that is required for SEK1 activation.

(*b*) *SEK Regulation*

Because the SAPK/SEK pathway was not activated by the same agonists as the MAPK pathway, Raf (and Mos) kinases were unlikely candidates to phosphorylate and activate SEK. Indeed, Raf does not phosphorylate SEK1 *in vitro*.

In budding and fission yeasts there are three protein kinases that act on MEK-like kinases, STE11, BYR1 and BCK1, which are structurally dissimilar from Raf (see figure 1). A mammalian homologue of the STE11 and BYR1 genes was isolated by polymerase chain reaction cloning. This protein kinase, when expressed at high levels in Cos cells, phosphorylated and activated MEK and was therefore termed MEK kinase (MEKK1) (Lange-Carter *et al.* 1993). Incubation of the constitutively active catalytic domain of MEKK1 with SEK resulted in rapid phosphorylation and activation of SAPK kinase activity. Furthermore, a mutant SEK1 containing Ala220 and Leu224 (SEK-AL) was not a substrate and was not activated (Yan *et al.* 1994). Co-expression of MEKK1 and SAPKα in HeLa cells resulted in potent activation of the latter.

These results presented a conundrum because the MAPK and SAPK pathways are differentially regulated yet MEKK1 apparently could activate both. However, when inducibly expressed at physiological levels in fibroblasts, MEKK1 was incapable of activating the MAPK pathway but retained stimulation of SAPKs (Yan *et al.* 1994). These data strongly suggest that MEKK1 is a physiological SEK kinase rather than a MEK kinase.

(*c*) *MEKK Regulation*

The catalytic domain of MEKK1 is constitutively active. The full length protein is 78–90 kDa in size and consists of an N-terminal 'regulatory' domain followed by the catalytic domain. Several mammalian MEKK genes have been isolated which primarily differ in their regulatory region. Unfortunately, even the full-length protein exhibits constitutive activity when transfected into cells which severely complicates analysis of its regulation. Furthermore, unlike inactive forms of MEK and SEK, kinase-dead MEKK1 does not exhibit a dominant negative effect.

In budding yeast STE11 has been placed genetically downstream of yet another protein kinase termed

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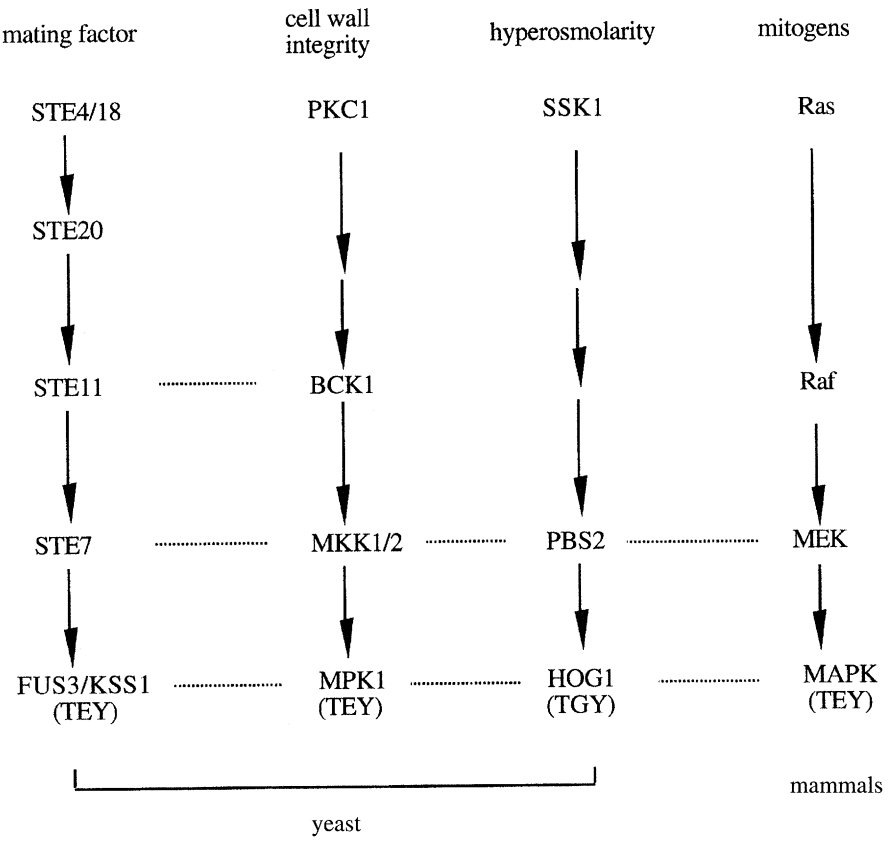

Figure 1. Parallel signalling pathways in yeast. Shown are the genetically identified components of three yeast signalling pathways. The arrows between these do not necessarily reflect a direct interaction, although several of the pairs of gene products have been demonstrated to associate in cells. By comparison, the MAPK pathway in mammals is shown. The broken horizonal lines indicate structurally related proteins.

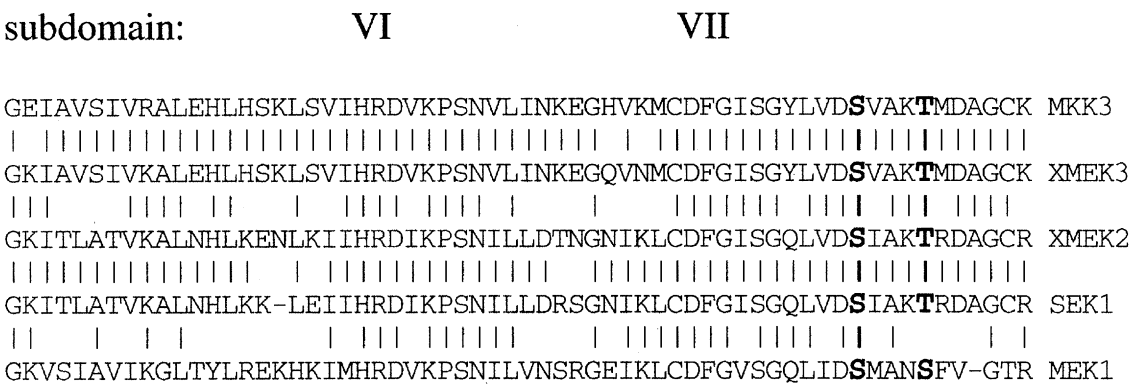

Figure 2. Comparison of amino acid sequences surrounding the activation domains of MEK-class protein kinases. Activating phosphorylation sites are in bold.

STE20 (Herskowitz 1995; see figure 3). Because MEKK1 is a phosphoprotein, a mammalian STE20 homologue is a reasonable candidate for an upstream regulator. However, it should be emphasized that although STE11, STE7 and FUS3/KSS1 genetically and biochemically interact, there may be intervening gene products between STE20 and STE11. With this caveat, we have examined the potential role for STE20 homologues as components of the SAPK pathway. Several protein kinase related to STE20 are known including PAK1, PAK2, GC kinase and two novel kinases (Manser *et al.* 1994; Katz *et al.* 1994; Martin *et al.* 1995). Whereas transfection of several of these protein kinases into Cos cells has no effect on SAPK activity, expression of GC kinase does activate SAPK in a SEK-dependent manner (Pombo *et al.* 1995).

What are the initial signalling components of the SAPK pathway? This pathway is induced by a wide variety of agents and it is likely that many of these act on independent cellular targets or 'sensors'. For example, UV irradiation induces cysteine cross-links thus dimerizing receptors and many other proteins causing their activation. The response to this agonist is therefore likely to be complex and to involve several

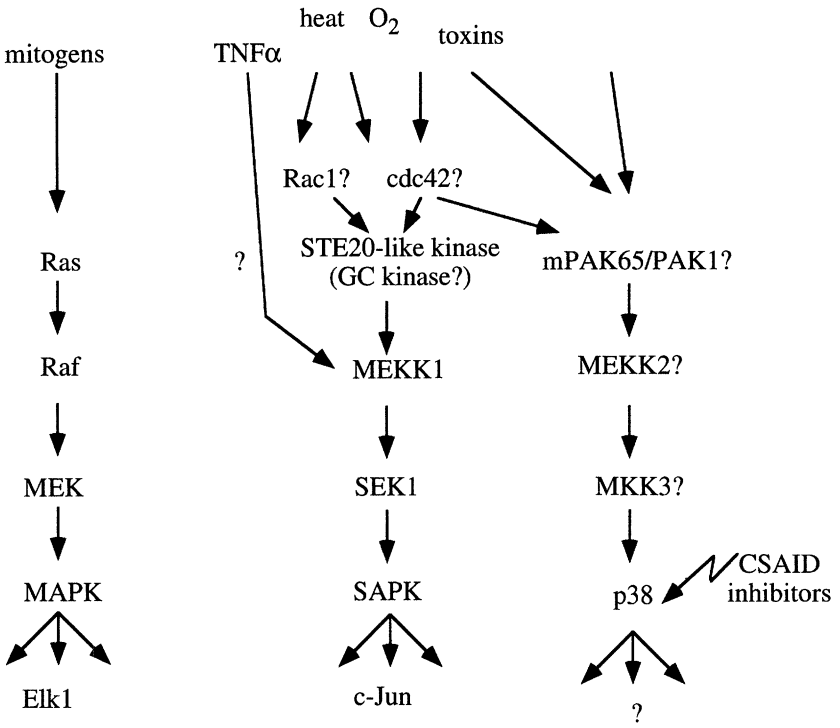

Figure 3. Mammalian MAPK pathways. Outlined are the components of the MAPK, SAPK and p38 pathways which largely respond to mitogens or stress agents respectively. The mechanisms via which MKK3 and MEKK are regulated and the identities of components upstream of these two protein kinases are currently unknown.

parallel pathways. The inflammatory cytokines such as TNF-α and IL-1 act via cell surface receptors. Although the mechanisms via which signalling systems are invoked by these receptors is unclear, they induce activation of sphingomyelinases (Weigmann *et al.* 1994). Addition of bacterial sphingomyelinase to cells in culture causes SAPK activation suggesting a role for this pathway in the cytokine response (Kyriakis *et al.* 1994).

In yeast, STE20 is downstream of heterotrimeric G proteins (STE4/18) that are coupled to the mating factor receptors. There is evidence for a role of small G proteins in SAPK regulation. For example, carbacol, acting via G protein-linked muscarinic acetylcholine receptors, is a potent SAPK agonist (Coso *et al.* 1995). More recently, cdc42 and Rac1 have been implicated activating PAK2 (Martin *et al.* 1995) and in inducing the SAPK pathway (Coso *et al.* 1995*a*).

The responses to heat, metabolic poisons, tunicamycin and hypoxia likely involve yet other mediators. For example, certain cell lines that exhibit thermotolerance are defective in heat activation of the SAPK pathway, but respond normally to other agonists suggesting the existence of distinct sensor elements for different stimuli (see figure 3).

3. THE P38 PATHWAY

The existence of multiple MAPK pathways in yeast led many groups to search for novel components of signalling pathways in mammals (see figure 3). Through the use of genetic complementation, for example, one of the SAPKs (JNK1) was shown to rescue a defect in a MAPK termed HOG1 which is activated by hyperosmolarity (Galcheva-Gargova *et al.* 1994). JNK1 is not the mammalian homologue of HOG1 however, as another protein kinase, termed p38, exhibits a higher degree of similarity to HOG1 including the presence of a glycine residue between the tyrosine and threonine activation sites (TGY; Han *et al.* 1994). The substrates of p38 are poorly characterized to date, but one likely target is MAPKAP kinase-2 (Rouse *et al.* 1994; Freshney *et al.* 1994)

p38 is activated by very similar agonists to the SAPKs and the kinase is phosphorylated by SEK1 *in vitro* . However, there are several differences in the spectrum of stimulants for SAPK and p38 and the time courses of activation are not identical. Furthermore, SEK1 does not interact with p38 in cells and is not activated following MEKK1 expression. These data rule out SEK1 as a physiological activator of p38 and suggest the existence of a distinct regulatory pathway. Recently, a novel MEK-like kinase was isolated termed MKK3 (Dérijard *et al.* 1995). This protein specifically phosphorylates p38 *in vitro* and when co-expressed in cells. However, the physiological relevance of p38 regulation by MKK3 remains to be established. Given the existence of multiple MEKK genes and the fact that MEKK1 does not activate p38, it is likely that MKK3 (or a related p38 kinase) will be directly regulated by a novel MEKK-like protein.

The biology of p38 was significantly advanced by the finding that a series of pyridinyl-imidazole compounds specifically bind and inhibit this protein kinase (Lee *et al.* 1994). Indeed, two splice variants of the protein kinase were isolated by virtue of the interaction. The compounds are bio-active as anti-inflammatory

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agents by virtue of their blockade of the secretion of inflammatory cytokines such as TNF-α and IL-1β. Because they appear to be highly specific for p38, the compounds (known as cytokine-suppressive anti-inflammatory drugs, CSAIDs) implicate this protein kinase in the pathway inducing cytokine production (Lee *et al.* 1994). One of the stimulants of the kinase is lipopolysaccharide (Han *et al.* 1994), a potent cytokine inducer and the moiety responsible for septic shock in response to pathogenic bacteria. Because p38 is also activated by inflammatory cytokines, it is likely to be involved in both the induction and response to these agents.

In addition to the two splice forms of p38 mentioned, another form termed Mxi2 was identified by a yeast interaction screen using the c-Myc binding partner, Max, as bait (A. Zerbos, personal communication). This form of the kinase has a helix-loop-helix (HLH) domain within its C-terminal region which mediates tight binding to the HLH region of Max. Presumably, this splice form is nuclear and specifically interacts with transcription factors, reminiscent of the SAPK/Jun association.

4. CONCLUSIONS AND PERSPECTIVES

The discovery of two novel signalling pathways which respond predominantly to cellular stress raises many questions concerning their physiological function. The pathways might trigger repair responses following damage thus acting in a reparative manner. Alternatively, they may signal a cell to arrest and induce apoptosis and therefore contribute to the toxicity of the stress agents. The pathways are also involved in non-stress signalling in response to specific agonists such as carbacol, CD28 and CD40 (D. Cantrell and E. Clark, personal communication).

A clue to the physiological role of the SAPK pathway derives from the effect of MEKK1 expression on cells. Whereas it has not been possible to derive stable cell lines expressing MEKK1, fibroblast cell lines expressing this gene under an inducible promoter undergo growth arrest following activation of the promoter (see figure 4). Therefore, exclusive activation of the SAPKs results in growth inhibition and possibly apoptosis which often occurs following exposure of cells to toxic conditions.

It is possible that MEKK1 activates pathways in addition to the SAPK system. To address this we have derived cell lines expressing dominant-negative variants of SEK1. These cells have blunted activation of the SAPKs in response to anisomycin and are being used to determine the requirement for SAPK activation in the response of cells to stress stimuli (R. A. Tibbles & J. R. Woodgett, unpublished data).

The high degree of specificity of the CSAIDs for p38 suggests that further drugs may be found which interact with other classes of MAPKs. Identification of such inhibitors would greatly enhance study of the physiological role of the MAPK related pathways. Furthermore, they would probably have therapeutic value as anti-cancer drugs or in blockade of reperfusion injury. Despite rapid progress since the identification of the MAPK pathways, our understanding of the mechanisms by which cells respond and react to their environment is still nascent. There are clearly additional components and pathways to be discovered, but given that the cells demonstrated conservatism in exploiting the 'three kinase' module, progress in defining the remaining transduction molecules will undoubtedly be rapid.

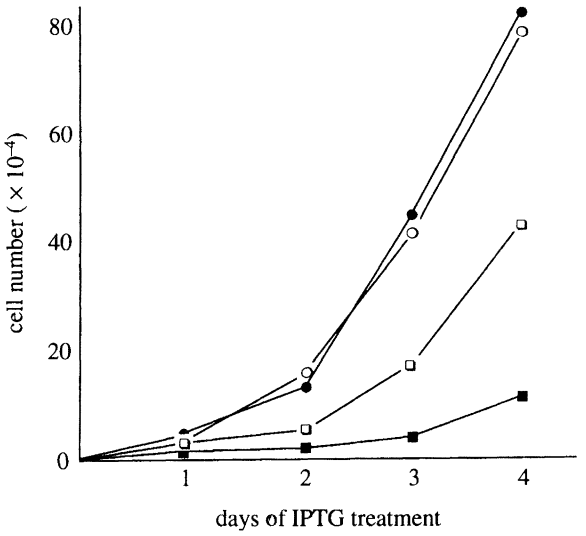

Figure 4. Effect of induction of MEKK on growth of fibroblasts. NIH3T3 cells (circles) or NIH3T3 cells expressing MEKK1 under the control of an IPTG-inducible promoter (squares) were treated with (filled symbols) or without (open symbols) 1 mM IPTG.

REFERENCES

Adler, V., Unlap, T. & Kraft, A. S. 1994 A peptide encoding the c-Jun δ domain inhibits the activity of a c-Jun amino-terminal protein kinase. *J. Biol. Chem.* **269**, 11186–11191.

Avruch J., Zhang X. F. & Kyriakis, J. M. 1994 Raf meets Ras: completing the framework of a signal transduction pathway. *TIBS* **19**, 279–83.

Bird, T. A., Kyriakis, J. M., Tyshler, L., Gayle, M., Milne, A. & Virca, G. D. 1994 Interleukin-1 activates p54 mitogen-activated protein (MAP) kinase/stress-activated protein kinase by a pathway that is independent of p21ras, Raf-1, and MAP kinase kinase. *J. Biol. Chem.* **269**, 31836–44.

Black, E. J., Catling, A. D., Woodgett, J. R., Kilbey, A. & Gillespie, D. A. F. 1994 Transcriptional activation by the v-Jun oncoprotein is independent of positive regulatory phosphorylation. *Oncogene* **9**, 2363–2368.

Boyle, W. B., Smeal, T., Defize, L. H. K. *et al.* 1991 Activation of protein kinase C decreases phosphorylation of cJun at sites that negatively regulate its DNA binding activity. *Cell* **64**, 573–584.

Cano, E. & Mahadevan, L. C. 1995 Parallel signal processing among mammalian MAPKs. *TIBS* **20**, 117–122.

Coso O. A., Chiariello, M., Kalinec, G., Kyriakis, J. M., Woodgett, J. & Gutkind J. S. 1995*a* Transforming G protein-coupled receptors potently activate JNK (SAPK). Evidence for a divergence from the tyrosine kinase signaling pathway. *J. Biol. Chem.* **270**, 5620–5624.

Coso, O. A., Chiariello, M., Yu, J. *et al.* 1995*b* The small GTP-binding proteins Rac1 and Cdc42 regulate the

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

activity of JNK/SAPK signaling pathway. *Cell* **81**, 1137–1146.

Cowley, S., Paterson, H., Kemp, P. & Marshall, C. J. 1994 Activation of MAP kinase kinase is necessary and sufficient for PC12 differentiation and for transformation of NIH3T3 cells. *Cell* **77**, 841–852.

Dai, T., Rubie, E. A., Franklin, C. C. *et al.* 1995 SAP kinases bind directly to the δ domain of c-Jun in resting cells: implications for repression of c-Jun function. *Oncogene* **10**, 849–855.

Dérijard, B., Hibi, M, Wu, I.-H. *et al.* 1994 JNK1: a protein kinase stimulated by UV light and Ha-Ras that binds and phosphorylates the c-Jun activation domain. *Cell* **76**, 1025–1037.

Dérijard, B., Raingeaud, J., Barrett, T. *et al.* 1995 Independent human MAP kinase signal transduction pathways defined by MEK and MKK isoforms. *Science*, *Wash.* **267**, 682–685.

Franklin, C. C., Sanchez, V., Wagner, F., Woodgett, J. R., & Kraft, A. S. 1992 Phorbol ester-induced amino terminal phosphorylation of c-Jun but not JunB regulates transcriptional activation. *Proc. natn. Acad. Sci. U.S.A.* **89**, 7247–7251.

Freshney, N. W., Rawlinson, L., Guesdon, F. *et al.* 1994 Interleukin-1 activates a novel protein kinase cascade that results in the phosphorylation of hsp27. *Cell* **78**, 1039–1049.

Galcheva-Gargova, Z., Dérijard, B., Wu, I.-H. & Davis, R. J. 1994 An osmosensing signal transduction pathway in mammalian cells. *Science*, *Wash.* **265**, 806–808.

Gupta, S., Campbell, D., Dérijard, B. & Davis, R. J. 1995 Transcription factor ATF2 regulation by the JNK signal transduction pathway. *Science*, *Wash.* **267**, 389–393.

Han, J., Lee, J.-D., Bibbs, L. & Ulevitch, R. J. 1994 A MAP kinase targeted by endotoxin and hyperosmolarity in mammalian cells. *Science*, *Wash.* **265**, 808–811.

Herskowitz, I. 1995 MAP kinase pathways in yeast: for mating and more. *Cell* **80**, 187–197.

Hill, C. S. & Treisman, R. 1995 Transcriptional regulation by extracellular signals: mechanisms and specificity. *Cell* **80**, 199–211.

Hou, X. S., Chou, T.-B., Melnick, M. B. & Perrimon, N. 1995 The torso tyrosine kinase can activate Raf in a Ras-independent pathway. *Cell* **81**, 63–71.

Katz, P., Whalen, G. & Kehrl, J. H. 1994 Differential expression of a novel protein kinase in human B lymphocytes. *J. Biol. Chem.* **269**, 16802–16809.

Kyriakis, A. & Avruch, J. 1990 A novel serine/threonine protein kinase regulated by phosphorylation and stimulated by poly-L-lysine. *J. Biol. Chem.* **265**, 17355–17363.

Kyriakis, J. M., Banerjee, P., Nikolakaki, E. *et al.* 1994 The stress-activated protein kinase subfamily of c-Jun kinases. *Nature*, *Lond.* **369**, 156–160.

Kyriakis, J. M., Brautigan, D. L., Ingebritsen, T. S. & Avruch, J. 1991 pp54 microtubule-associated protein-2 kinase requires both tyrosine and serine/threonine phosphorylation for activity. *J. Biol. Chem.* **266**, 10043–10046.

Lange-Carter, C. A., Pleiman, C. M., Gardner, A. M., Blumer, K. J. & Johnson, G. L. 1993 A divergence in the MAP kinase regulatory network defined by MEK kinase and Raf. *Science*, *Wash.* **260**, 315–319.

Lee, J. C., Laydon, J. T., McDonnell, P. C., Gallagher, T. F. *et al.* 1994 A protein kinase involved in the regulation of infammatory cytokine biosynthesis. *Nature*, *Lond.* **372**, 739–745.

Livingstone, C., Patel, G. & Jones, N. 1995 ATF-2 contains a phosphorylation-dependent transcriptional activation domain. *EMBO J.* **14**, 1785–1797.

Lloyd, A., Yancheva, N. & Wasylyk, B. 1991 Transformation suppressor activity of a Jun transcription factor lacking its activation domain. *Nature*, *Lond.* **352**, 635–638.

Manser, E., Leung, T., Salihuddin, H., Zhao, Z. & Lim, L. 1994 A brain serine/threonine protein kinase activated by Cdc42 and Rac1. *Nature*, *Lond.* **367**, 40–49.

Martin, G. A., Bollag, G., McCormick, F. & Abo-A. 1995 A novel serine kinase activated by rac1/CDC42Hs-dependent autophosphorylation is related to PAK65 and STE20. *EMBO J.* **14**, 1970–1978.

Minden, A., Lin, A., McMahon, M. *et al.* 1994 Differential activation of ERK and JNK mitogen-activated protein kinases by Raf-1 and MEKK. *Science*, *Wash.* **266**, 1719–1723.

Mohit, A. A., Martin, J. H. & Miller, C. A. 1995 p493F12 kinase: a novel MAP kinase expressed in a subset of neurons in the human nervous system. *Neuron* **14**, 67–78.

Moriguchi, T., Kawasaki, H., Matsuda, S., Gotoh, Y. & Nishida, E. 1995 Evidence for multiple activators for stress-activated protein kinases/c-Jun amino-terminal kinases. Existence of novel activators. *J. Biol. Chem.* **270**, 2969–2977.

Neiman, A. M. 1993 Conservation and reiteration of a kinase cascade. *TIG* **9**, 390–394.

Nikolakaki, E., Coffer, P., Hemelsoet, R., Woodgett, J. R., & Defize, L. H. K. 1993 Glycogen synthase kinase-3 phosphorylates Jun-family members *in vitro* and negatively regulates their transactivating potential in intact cells. *Oncogene* **8**, 833–840.

Pombo, C. M., Bonventre, J. V., Woodgett, J. R., Kyriakis, J. M. & Force, T. 1994 The stress-activated protein kinases (SAPKs) are major c-Jun amino terminal kinases activated by ischemia and reperfusion. *J. Biol. Chem.* **269**, 26546–26550.

Pombo, C. M., Kherl, J. H., Sanchez, I., Katz, P., Avruch, J., Zon, L. I., Woodgett, J. R., Force, T. & Kyriakis, J. M. 1995 Activation of the SAPK pathway by the human STE20 homologue germinal centre kinase. *Nature*, *Lond.* **377**, 750–754.

Pulverer, B., Kyriakis, J., Avruch, J., Nikolakaki, H. & Woodgett, J. R. 1991 Phosphorylation of c-*jun* by MAP kinases. *Nature*, *Lond.* **353**, 670–674.

Rouse, J., Cohen, P., Trigon, S. *et al.* 1994 A novel kinase cascade triggered by stress and heat shock that stimulates MAPKAP kinase-2 and phosphorylation of the small heat shock proteins. *Cell* **78**, 1027–1037.

Sanchez, I., Hughes, R., Mayer, B. *et al.* 1994 SAP/ERK kinase-1 (SEK1) defines the SAPK pathway regulating c-Jun N-terminal phosphorylation. *Nature*, *Lond.* **372**, 794–798.

Smeal, T., Binetruy, B., Mercola, D. A., Birrer, M. & Karin, M. 1991 Oncogenic and transcriptional cooperation with Ha-Ras requires phosphorylation of c-Jun on serines 63 and 73. *Nature*, *Lond.* **354**, 494–496.

Van Dam, H., Wilhelm, D., Herr, I., Steffen, A., Herrlich, P. & Angel, P. 1995 ATF-2 is preferentially activated by stress-activated protein kinases to mediate c-Jun induction in response to genotoxic agents. *EMBO J.* **14**, 1798–1811.

Weigmann, K., Schütze, S., Machleidt, T., Witte, D. & Krönke, M. 1994 Functional dichotomy of neutral and acidic sphingomyelinases in tumor necrosis factor signaling. *Cell* **78**, 1005–1015.

Yan, M., Dai, T., Deak, J. *et al.* 1994 MEKK1 activates the stress activated protein kinase (SAPK) *in vivo*, not MAP kinase, via direct phosphorylation of the SAPK activator SEK1. *Nature*, *Lond.* **372**, 798–800.

Yashar, B. M., Kelley, C., Yee, K., Errede, B. & Zon, L. I. 1993 Novel members of the mitogen-activated protein kinase activator family in *Xenopus laevis*. *Molec. Cell Biol.* **13**, 5738–5748.

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Discussion

Question. Is activation of the protein kinase p38Hog affected by dominant-negative SEK1 in cells?

J. R. Woodgett. If we look in COS cells that massively over-express SEK-AL then phosphorylation of p38 is reduced by about a quarter. In the SEK-AL lines, the Hog pathway is unchanged. Some of these effects in COS cells may occur because massive over-expression of components make them influence pathways in which they normally would not participate. For example, over-expression of MEKK in COS cells effectively activates the MAPK pathway, but if expression levels are lower then the main influence is on the SAPK pathway.

Question. What are likely to be the relative contributions in these pathways of heterodimers including Fos and ATF2, compared to Jun:Jun homodimers or Jun:ATF2 heterodimers?

J. R. Woodgett. A number of us have shown that both Jun and ATF2 are substrates for these kinases, but there is little or no direct evidence that Fos is such a substrate. There is also evidence from *in vitro* studies that different homodimers and heterodimers can be phosphorylated to different degrees, but the available evidence on intact cells makes it very difficult to determine the relative substrate specificities of the kinases *in situ*. For example, in F9 teratocarcinoma cells, which have very little intrinsic AP1, Jun activation is not prevented by the expression of mutant forms of Fos.

J. L. Bos (*Utrecht University, The Netherlands*). There seem to be clear disagreements between Dr Woodgett, Professor Karin and others as to the involvement of Ras in the control of these pathways. What does Dr Woodgett think is the true situation?

J. R. Woodgett. There certainly can be fairly small stimulatory effects of Ras on these pathways, which can particularly be shown by using dominant-negative Ras mutants. However, these tend to be quite small effects and one needs to remember that interfering with as important a cell switch as Ras can also have indirect effects. For example, it is sometimes possible to produce modest inactivation of the pathways with dominant-negative Ras proteins, but then see no clear activation when the same cells are transfected with constitutively active Ras. If one compares the small effects of active mutants of Ras with any of the stress agonists that I have been discussing, the difference is like night and day: the stress agonists produce very large effects and Ras only small effects.

M. Karin (*Department of Pharmacology, University of California, San Diego, U.S.A.*). I do not think that our results and those of Dr Woodgett are very different. The difference is that we tend to focus on cell lines in which we obtain good effects with Ras and growth factors and to study those. We have not spent much effort on cell lines in which we do not see a nice response to growth factors or Ras. For example, one can see very good responses to EGF in HeLa cells.

C. J. Marshall (*Chester Beatty Laboratory, Institute of Cancer Research, London, U.K.*). Maybe one can partly reconcile these problems by considering the way in which Raf-1 is activated. Activated Ras recruits Raf-1 to the plasma membrane as a part of a complex activation process that also includes tyrosine phosphorylation. Maybe dominant negative Ras can block the entire pathway, but over-expression of constitutively active Ras only provides one of the signals needed for activation, so that such active forms of Ras will not fully activate the downstream pathway.

M. Karin. Indeed, in Junkat T cells we have already shown that Ras activation alone results in weak JNK activation. This response is strongly potentiated by elevating intracellular Ca^{2+} or activation of the CD28 coreceptor. We are still searching for the coactivating signals in other cell types.

R. Treisman (*Imperial Cancer Research Fund, London, U.K.*). I keep hearing rumours that the MEK kinase clone is not complete. Is this true?

J. Woodgett. It is obviously unfair to comment extensively on the work of others, but I will. The original Gary Johnson MEK kinase was a 70 kDa protein. However, Melanie Cobb now has a 130 kDa clone. The experiments I showed you were done either with a 94 kDa clone or with a shorter kinase domain clone of approximately 40 kDa; the results obtained with both are the same. However, the fact that they are both very active makes studying their regulation very difficult. Melanie Cobb's larger protein is said to be more highly regulatable, but if you use the available MEKK antibodies the main transcript that you pick up in most cells of 87–92 kDa, so the possible role of the larger form in cells remains in question.

In addition, there are obviously multiple MEKKs. Last year, for example, Gary Johnson reported in *Science* that NGF or EGF can activate MEKK in PC-12 cells, but the enzyme involved was not recognized by MEKK-1 anti-peptide antibodies. Various groups now seem to have evidence for three or four other MEKKs, which are presumably differentially regulated.

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