Mammalian Mitogen-activated Protein Kinase Pathways Are Regulated through Formation of Specific Kinase-Activator Complexes*

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Mammalian cells contain at least three signaling systems which are structurally related to the mitogen-activated protein kinase (MAPK) pathway. Growth factors acting through Ras primarily stimulate the Raf/MEK/MAPK cascade of protein kinases. In contrast, many stress-related signals such as heat shock, inflammatory cytokines, and hyperosmolarity induce the MEKK/SEK-MKK4/SAPK(JNK) and/or the MKK3 or MKK6/p38hog pathways. Physiological agonists of these pathway types are either qualitatively or quantitatively distinct, suggesting few common proximal signaling elements, although past studies performed in vitro, or in cells using transient over-expression, reveal interaction between the components of all three pathways. These studies suggest a high degree of cross-talk apparently not seen in vivo. We have examined the possible molecular basis of the differing agonist profiles of these three MAPK pathways. We report preferential association between MAP kinases and their activators in eukaryotic cells. Furthermore, using the yeast 2-hybrid system, we show that association between these components can occur independent of additional eukaryotic proteins. We show that SAPK(JNK) or p38hog activation is specifically impaired by co-expression of cognate dominant negative MAP kinase mutants, demonstrating functional specificity at this level. Further divergence and insulation of the stress pathways occurs proximal to the MAPK kinases since activation of the MAPK kinase MEKK results in SAPK(JNK) activation but does not cause p38hog phosphorylation. Therefore, in intact cells, the three MAPK pathways may be independently regulated and their components show specificity in their interaction with cognate cascade members. The degree of intermolecular specificity suggests that mammalian MAPK signaling pathways may remain distinct without the need for specific scaffolding proteins to sequester components of individual pathways.

Genetic studies in yeast first revealed the existence of multiple distinct mitogen-activated protein kinase (MAPK) related signal transduction pathways containing structurally similar protein kinase cascades mediating responses to mating factor, hyperosmolarity, and cell wall integrity (1). Pathways in mammals, which likely have different physiologic significance, have been discovered which utilize related protein kinase cascades (2–4). The archetypal MAPK pathway is activated in response to Ras-GTP loading as well as other processes such as phosphatidylinositol turnover (5–7). The pathway comprises a series of protein kinases such that activation of the Raf proto-oncogene causes phosphorylation and activation of MEK which, in turn, phosphorylates and activates the MAPKs, Erk1, and -2 (4, 8). The targets of these kinases include other protein kinases and transcription factors such as p62 hog (9–11). While mitogens and growth factors commonly stimulate this MAPK pathway, cells respond to cellular stress agents by induction of two structurally related but distinct pathways (2, 12–16). Stress stimuli such as the inflammatory cytokines, tumor necrosis factor α, and interleukin-1, thermal shock, ischemia/reperfusion, and hyperosmolarity cause activation of two recently described groups of MAPK-related proteins, the SAPK/JNK family and p38 hog kinase (2, 12, 16, 17). SAPK is phosphorylated by a MEK-like kinase termed SEK1 (MKK4, JNKK), while p38 hog is phosphorylated by the related kinases MKK3 and MKK6 (18–20). SEK1 itself is phosphorylated and activated by MEKK (21) while MKK3 and MKK6 activators are being defined.

Activation of these three pathways is not mutually exclusive. For example, heat shock and tumor necrosis factor α partially activate the ERK1/ERK2 cascade and some mitogens such as epidermal growth factor partially activate the SAPK pathway (2, 12). The mitogenic and stress pathways are, however, discriminated by agonist profiles when efficiencies of activation by a broad range are compared (12). The functional selectivity of varying agonists may not be manifest in vitro or when signaling proteins are overexpressed in cells by transient transfection. For example, MEKK was first characterized as a protein kinase acting on MEK (22). At high levels, this enzyme does phosphorylate and activate MEK but at physiological levels MEKK specifically stimulates the SAPK pathway through SEK phosphorylation (21). Recently, SEK has been shown to phosphorylate p38 hog in vitro and in transfected COS cells raising the possibility that SAPK and p38 hog are co-regulated by this kinase (23). To address the discrepancy between agonist discrimination and apparent in vitro promiscuity, we have screened a variety of conditions for induction of these pathways and have probed for both physical association between the components in intact eukaryotic cells or in yeast cells, and for functional relationships using a spectrum of dominant negative MAP kinase mutants. These results indicate that the
three pathways operate independently in cells due to effective compartmentalization of their components via specific protein-protein interactions.

**EXPERIMENTAL PROCEDURES**

**Mutagenesis—**Mammalian SEK was mutated at serines 204 and 207, the sites of activating phosphorylation, using the pSelect system (Promega) and the protocol supplied by the manufacturer. The alanine 220, leucine 224 mutant (SEK-AL) was generated with the oligonucleotide GCTTTGAGCCTATTTGCAAGTTAGATGC. MKK3 was mutated at serine 231 to alanine and at threonine 235 to leucine using the oligonucleotide TGGTGGAGCTTGGCCAAAGCTTATAGGATCGGC, producing MKKK-AL. Serine 207 and threonine 211 of MKK6 were mutated to alanine and leucine to produce MKKK6-AL using the oligonucleotide ACTTTGGAGCCTATTTGCAAGTTAGATGCAGGT- TGCACAAA.

**Plasmid Vectors—**The complete open reading frames of mammalian ERK-2, SAPKs, and p38\(^{\text{\text{hog}}}\) kinases were cloned in-frame at the carboxyl terminus of the glutathione S-transferase gene in the eukaryotic expression vector pEBG (19). Amino-terminal epitope-tagged versions of wild-type (wtSEK, SEK-AL, wtMKK3, MKK3-AL, wtMKK6, MKK6-AL, MEKK, and MEK) were constructed using the 13-amino acid influenza hemagglutinin sequence (HA), MGYPYDPYDYG. These HA-tagged proteins were expressed under the control of the adenovirus major late promoter in the SV40ori containing plasmids pMT2 (19) or pcDNA3 (Invitrogen, San Diego, CA).

**Tissue Culture, Gene Expression, and Cell Stimulation—**Calcium phosphate coprecipitations were performed as previously described (4). RIF-1 murine fibroblasts (gift of Dr. G. Hahn, Stanford University) were transfected with pcDNA HA/SEK-AL and selected in 300 \(\mu\)g/ml G418. Individual drug-resistant colonies were selected after 2 weeks of incubation and analyzed for expression of tagged proteins by immunoprecipitation followed by Western blot analysis using 12CA5 tissue culture supernatant. MKKK6-AL or wtMKK6s were transiently transfected into COS cells with either pEBG SAPK or pEBG p38\(^{\text{\text{hog}}}\) using the same technique. COS cells, NIH 3T3 fibroblasts, or Jurkat lymphoblasts were maintained in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) containing 10% fetal calf serum (Life Technologies, Inc.). On the day prior to stimulation cells were serum starved for 16 h in the same medium containing 2.0% fetal calf serum. Stimulation, when performed, was with a 10-min exposure to gemicidal UV light (254 nm (General Electric)) or 30 min exposure to 500 \(\mu\)g/ml anisomycin, 0.5 \(\mu\)m sodium arsenite, 400 \(\mu\)m sorbitol, 10 \(\mu\)m phorbol 12-myristate 13-acetate (all from Sigma) or to heat shock for 30 min at 42.0 \(^{\circ}\)C.

**Immunoprecipitation and Glutathione-Sepharose Affinity Protein Purification—**For immunoprecipitation, co-immunoprecipitations, and glutathione-Sepharose affinity protein purification—For immunoprecipitation, co-immunoprecipitations, and glutathione-Sepharose affinity protein purification, cells were incubated in Nonidet P-40 lysis buffer consisting of 150 mM NaCl, 1% Nonidet P-40, 10 mM Tris–HCl (pH 8.0) and 1 mM phenylmethylsulfonylfluoride (Sigma). Phosphatase activity was inhibited by 5 \(\mu\)M sodium orthovanadate and 50 \(\mu\)M dithiothreitol (Sigma). HA epitope-tagged proteins were immunoprecipitated for 1 h at 4 \(^{\circ}\)C using supernatant from the 12CA5 hybridoma (Boehringer Mannheim) preincubated with protein A-Sepharose (Pharmacia) for 20 min at room temperature. GST fusion proteins were purified by incubation with glutathione-Sepharose affinity resin (Pharmacia) at 4 \(^{\circ}\)C for 1 h. Immunoprecipitates and glutathione-Sepharose resin purified proteins were washed 3 times with lysis buffer.

**In Vitro Kinase Reaction—**In vitro kinase reactions were performed using immunoprecipitated bead immobilized native p38\(^{\text{\text{hag}}}\) or SAPK or affinity purified bead immobilized GST tagged versions of the same proteins. Each kinase reaction was performed in a final volume of 50 \(\mu\)l containing 25 \(\mu\)l of 2 \(\times\) kinase buffer (100 \(\mu\)M ATP, 10 mM MgCl\(_2\), 50 mM Tris-Cl (pH 7.5), 1 mM EDTA, 5 \(\mu\)l of myelin basic protein (1 mg/ml) or calf-thymus DNA (1 mg/ml) dissolved in distilled water and 20 \(\mu\)l of immobilized kinase slurry. Samples were incubated at 37 \(^{\circ}\)C for 30 min. Reactions were terminated by the addition of 50 \(\mu\)l of 2 \(\times\) SDS protein loading buffer followed by boiling for 10 min. Products were separated on an 8% polyacrylamide gel followed by transfer to 3MM paper and autoradiography.

**Image Analysis—**Radiolabeled proteins were detected post-electrophoresis by PhosphorImager and the ImageQuant analysis software (Molecular Dynamics Inc., Sunnyvale CA).

**Yeast 2-Hybrid Analysis—**The complete coding sequences of SAPKs, p38\(^{\text{\text{hog}}}\), or p44\(^{\text{\text{hog}}}\) were expressed as GAL4 DNA-binding domain fusions in the plasmid pAS1 (Clonetech Inc.) while cDNAs coding SEK1, MEK, MKK3, and MKK6s were expressed as GAL4 trans-activating domain fusions using the vector pACTII (Clonetech Inc.). Expression of each fusion protein was confirmed by plasmid transfection into yeast strain HD7c followed by Western analysis of nutritionally selected colonies using antibodies specific for either the GAL4 DNA binding or transactivating domain. Co- transformation of HD7c with each pAS1 construct and each of the pACTII constructs was then performed. Control co- transformations of the same strain was performed using the vector of the pAS1 kinase constructs with empty pACTII. Co-transfected yeast were maintained prior to testing on minimal growth medium lacking amino acids tryptophan and leucine. Filter lift assays for \(\beta\)-galactosidase production, as described by the manufacturer, were performed for each transfected yeast strain after growth on tryptophan/leucine-deficient medium. Yeast growth and the pigmentation of lifted filters were documented by photography. All experiments were done at least 5 times.

**RESULTS**

**Differential Activation of MAPK Homologues—**By assaying the activation state of the ERKs and SAPKs we have previously shown that agonists of these pathways are largely distinct (19). These studies did not evaluate the activation of p38\(^{\text{\text{hag}}}\), which has been shown by others to be stimulated by agonists similar to those activating the SAPKs (16, 17), suggesting that the p38\(^{\text{\text{hag}}}\) pathway may share upstream components with the SAPK pathway. To test this we first directly compared activation of both SAPK and p38\(^{\text{\text{hag}}}\) which were immunoprecipitated after stimulation of either NIH 3T3 cells or Jurkat lymphocytes. Activity of immunoprecipitated SAPK was assayed using its physiologic substrate c-Jun, while the artificial substrate myelin basic protein was used to assay the activation state of immunoprecipitated p38\(^{\text{\text{hag}}}\). As shown in Fig. 1, activation of each of these kinases was both agonist and cell type dependent. p38\(^{\text{\text{hag}}}\) activity was stimulated only by UV irradiation and hyperosmolar shock (sorbitol) in Jurkat cells and by these agonists and phorbol 12-myristate 13-acetate, heat shock, and sodium arsenite in NIH 3T3 cells. In contrast, SAPK activity was stimulated by hyperosmolality, heat, and anisomycin in Jurkat cells and by all the agonists tested in NIH 3T3 cells. Of note, anisomycin stimulated SAPK but not p38\(^{\text{\text{hag}}}\) in both cell types. Sodium arsenite stimulated p38\(^{\text{\text{hag}}}\) in both cell types but stimulated SAPK only in NIH 3T3 cells while UV irradiation stimulated SAPK only in NIH 3T3 cells but stimulated p38\(^{\text{\text{hag}}}\) in both cell types tested.

**MEKK Overexpression Does Not Result in in Vivo Phosphorylation of p38\(^{\text{\text{hag}}}\)—**Since many of the agonists which stimulate the SAPK pathway stimulate p38\(^{\text{\text{hag}}}\), these enzymes may share common upstream activators. SEK1, which has been shown to activate SAPK in vivo, phosphorylates both SAPK and p38\(^{\text{\text{hag}}}\) in vitro (23), implying that SEK1 may be common to both pathways in vivo. MEKK, the physiologic activator of SEK1, would then be expected to activate both SAPK and p38\(^{\text{\text{hag}}}\) by

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phosphorylation after transient overexpression. Phosphorylation of both by SEK1 would explain the similarity of agonist activation profiles of these pathways. pEBG/p38\textsuperscript{hog} was transiently transfected alone or with pMT2/MEKK into COS cells. Samples expressing GST/p38\textsuperscript{hog} were either left unstimulated or incubated with sorbitol, to demonstrate the ability of p38\textsuperscript{hog} to become phosphorylated under these experimental circumstances. GST/p38\textsuperscript{hog} was purified on glutathione beads from each cell sample. p38\textsuperscript{hog} phosphorylation on tyrosine, indicative of activation, was assessed by anti-phosphotyrosine immunoblotting of the purified products. Fig. 2 shows that while sorbitol stimulation results in the expected tyrosine phosphorylation of p38\textsuperscript{hog}, co-expression of MEKK had no stimulating effect. To directly compare the enzymatic activity of either SAPK or p38\textsuperscript{hog} after MEKK overexpression, pEBG/SAPK or pEBG/p38\textsuperscript{hog} were transiently expressed in COS cells alone or with pMT2/MEKK. GST/SAPK or GST/p38\textsuperscript{hog} were affinity purified on glutathione beads in each case and enzymatic activity was assessed in vitro against c-Jun for SAPK or myelin basic protein for p38\textsuperscript{hog}. Fig. 3 shows that SAPK, as expected, was stimulated by MEKK co-expression to a level equivalent to that induced by hyperosmolarity. p38\textsuperscript{hog}, however, was not stimulated by MEKK co-expression but only by the hyperosmolar stimulus.

**SEK1 Co-precipitates from Cells with SAPK But Not with p38\textsuperscript{hog}**—MEKK, a strong activator of SEK1, does not appear to cause enzymatic activation or tyrosine phosphorylation of p38\textsuperscript{hog}. This observation, which is in variance with the reported ability of SEK1 to phosphorylate p38\textsuperscript{hog} in vitro, suggests that SEK1 does not activate or associate with p38\textsuperscript{hog} in cells. We have previously observed a tight in vivo interaction between SEK1 and SAPK suggesting physiologic relevance (19). These observations were extended to evaluate potential in vivo association between SEK1 and p38\textsuperscript{hog}. pEBG/SAPK or pEBG/p38\textsuperscript{hog} were transiently expressed in COS cells with pMT2/HA-SEK. The GST proteins were affinity purified, washed on glutathione beads, and divided for analysis. Relative expression of SAPK and p38\textsuperscript{hog} was determined by anti-GST immunoblotting using half of each sample (Fig. 4A). GST/SAPK appears to be under expressed relative to GST/p38\textsuperscript{hog} in this typical experiment. Associating HA-SEK1 was detected in the remaining half of each sample by immunoblotting with the

![Figure 1](image1.png)

**Fig. 1. p38\textsuperscript{hog} and SAPK from two cell lines demonstrate distinct patterns of activation in response to stress agonists.** NIH 3T3 cells or Jurkat lymphocytes were incubated with phorbol 12-myristate 13-acetate, sorbitol, sodium arsenite, or anisomycin or exposed to either UV irradiation or heat shock as described. Native p38\textsuperscript{hog} or SAPK were immunoprecipitated using specific rabbit antisera and purified on protein A-Sepharose beads. In vitro kinase reactions were performed to assess activity for each immunoprecipitate using the substrates myelin basic protein for p38\textsuperscript{hog} and GST-Jun for SAPK. The results were analyzed by acrylamide gel electrophoresis followed by PhosphorImaging. Densitometric determinations in each lane are provided and allow comparison within each panel.

![Figure 2](image2.png)

**Fig. 2. MEKK overexpression does not result in p38\textsuperscript{hog} phosphorylation.** COS cells were transiently co-transfected with both pEBG/p38\textsuperscript{hog} and pMT2/MEKK expression plasmids. Similar cell samples were transfected with pEBG/p38\textsuperscript{hog} alone and left unstimulated or were treated with 400 mM sorbitol for 30 min. GST/p38\textsuperscript{hog} was purified from each set of cells on glutathione-conjugated Sepharose. Tyrosine phosphorylation of the product, a measure of activation, was analyzed by acrylamide gel electrophoresis followed by anti-phosphotyrosine Western blotting. Data are shown in duplicate.

![Figure 3](image3.png)

**Fig. 3. MEKK overexpression enhances the activity of SAPK but not of p38\textsuperscript{hog}.** pMT2/MEKK and either pEBG/p38\textsuperscript{hog} or pEBG were transiently transfected into COS cells and either left unstimulated or stimulated with 400 mM sorbitol for 30 min. Control COS cells were transfected with either pEBG/p38\textsuperscript{hog} or pEBG alone and left unstimulated. GST/p38\textsuperscript{hog} or GST/SAPK were purified on glutathione-conjugated Sepharose. In vitro kinase reactions were performed to assess activity for each immunoprecipitate using the substrates myelin basic protein for p38\textsuperscript{hog} and GST-Jun for SAPK. The results were analyzed by acrylamide gel electrophoresis followed by autoradiography.

12CA5 monoclonal which is specific for the HA tag (Fig. 4B). Despite the underexpression of SAPK, under these experimental conditions, SEK1 binds to SAPK but not to p38\textsuperscript{hog}. This demonstrates specificity of SEK1 association with SAPK and suggests that p38\textsuperscript{hog} is unlikely to be activated by SEK1 in vivo although in vitro phosphorylation has been demonstrated. In a separate control experiment, pMT2/HA-MEK was expressed with pEBG/SAPK, pEBG/p38\textsuperscript{hog}, or pEBG/MAPK. Similarly, glutathione-Sepharose associating proteins were purified and analyzed by Western immunoblotting using the 12CA5 monoclonal antibody. Fig. 4C shows, as expected, that HA-MEK associates only with GST/MAPK but not with either GST/SAPK or GST/p38\textsuperscript{hog}, thus confirming the specificity of co-association observed under these experimental conditions.

**SEK-AL Expression Impairs SAPK But Not p38\textsuperscript{hog} Activation—SEK is activated by MEKK by phosphorylation on serine 204 and serine 207. We have mutated these residues to alanine and leucine, generating a dominantly active inhibitor of wild type SEK1 (19). Stable transfectants expressing SEK-AL in the murine fibrosarcoma cell line RIF-1 (24) were generated by G418 drug selection for pcDNA3/HA-SEK-AL plasmid incorporation. Two such lines, RIF/SEK-AL18 and RIF/SEK-AL19 were selected for further study. Samples of vector transfected RIF-1 and of the two SEK-AL expressing lines were left unstimulated or treated with 400 mM sorbitol or anisomycin. Endogenous SAPK or p38\textsuperscript{hog} were immunoprecipitated and activity of each kinase was directly evaluated in vitro. Fig. 5 demonstrates that while endogenous SAPK activation by ani-
the level of expression of the GST fusion proteins encoded by the pEBG vector were incubated with glutathione beads, washed, and the bead-associated proteins immunoblotted with anti-GST antibodies to determine specific association of signaling components.

Fig. 4. Specific association of signaling components. In panels A and B, COS cells were co-transfected for 48 h with pMT2/HA-SEK1 together with either pEBG/SAPK (lanes 2 and 4) or pEBG-p38<sub>hog</sub> (lanes 1 and 3). Samples from lanes 1 and 2 were from untreated cells while those in lanes 3 and 4 were from sorbitol-treated cells. In A, the lysates were incubated with glutathione beads, washed, and the bead-associated proteins immunoblotted with anti-GST antibodies to determine the level of expression of the GST fusion proteins encoded by the pEBG plasmids. In B, the lysates were directly immunoblotted with the monoclonal antibody 12CA5, specific for the HA epitope to detect associating tagged SEK1. In panel C, COS cells were co-transfected with pMT2/HA-MEK together with pEBG-SAPK (lane 1), pEBG-p38<sub>hog</sub> (lane 2), or pEBG-MAPK (lane 3). As in panel A, the GST fusion proteins were isolated and associating proteins were probed with 12CA5 antibody to detect the presence of HA-MEK. Detection was by enhanced chemiluminescence (ECL).

Fig. 5. Expression of the dominantly active inhibitor of SAPK activation, SEK-AL, does not inhibit p38<sub>hog</sub> activation in response to hyperosmolar shock. Two independently derived RIF-1 murine fibrosarcoma cell line clones expressing SEK-AL were derived utilizing G418 selection. A, SAPK activation in response to the stress agonist anisomycin and p38<sub>hog</sub> activation after sorbitol hyperosmolar shock was assessed by immunoprecipitating each native kinase followed by in vitro kinase reactions to assess activity as described above. Results were assessed by PhosphorImager and quantitated using the ImageQuant software package. B, graphical representation of p38<sub>hog</sub> and SAPK activation in each cell line and in vector transfected G418-resistant control cells.

somycin is impaired in the SEK-AL expressing cell lines, no impairment in the activation of p38<sub>hog</sub> is observed. The degree of substrate phosphorylation in Fig. 5A, an indication of activation, is represented graphically by densitometry in Fig. 5B.

MKK3-AL and MKK6-AL Expression Impair the Activation of p38<sub>hog</sub> But Not SAPK—MKK3 and MKK6 can phosphorylate p38<sub>hog</sub> in vitro and have been proposed as potential physiologic p38<sub>hog</sub> activators (18–20). MKK3 is regulated by phosphorylation on serine 189 and threonine 193, while MKK6 is phosphorylated on serine 207 and threonine 211, sites that are conserved among the other MAPK kinases SEK1 and MEK. Sites of serine phosphorylation were mutated to alanine and threo-nine phosphorylation sites were mutated to leucine in MKK3 and MKK6 using site-directed mutagenesis, forming MKK3-AL and MKK6-AL. MKK3-AL, MKK6-AL, and their wild type forms were HA epitope-tagged and each inserted into the vector pMT2. COS cells were transiently transfected with pEBG/p38<sub>hog</sub> or pEBG/SAPK and with empty pMT2 vector, pMT2/MKK3-AL, pMT2/MKK3-WT, pMT2/MKK6-AL, or pMT2/MKK6-WT. Samples of transfected cells were either unstimulated or incubated with anisomycin or sorbitol. GST/p38<sub>hog</sub> or GST/SAPK were affinity purified on glutathione beads and enzymatic activity was evaluated by in vitro kinase reactions. In Fig. 6A and B, graphical representation of p38<sub>hog</sub> and SAPK activity were markedly stimulated by anisomycin or by sorbitol which was not changed by the co-expression of MKK3-AL, MKK3-WT, MKK6-AL, or MKK6-WT. GST/p38<sub>hog</sub> or GST/SAPK were affinity purified on glutathione beads and enzymatic activity was evaluated by in vitro kinase reactions. In Fig. 6A and B, graphical representation of p38<sub>hog</sub> and SAPK activity were markedly stimulated by anisomycin or by sorbitol which was not changed by the co-expression of MKK3-AL, MKK3-WT, MKK6-AL, or MKK6-WT. GST/p38<sub>hog</sub> or GST/SAPK were affinity purified on glutathione beads and enzymatic activity was evaluated by in vitro kinase reactions. In Fig. 6A and B, graphical representation of p38<sub>hog</sub> and SAPK activity were markedly stimulated by anisomycin or by sorbitol which was not changed by the co-expression of MKK3-AL, MKK3-WT, MKK6-AL, or MKK6-WT. GST/p38<sub>hog</sub> or GST/SAPK were affinity purified on glutathione beads and enzymatic activity was evaluated by in vitro kinase reactions. In Fig. 6A and B, graphical representation of p38<sub>hog</sub> and SAPK activity were markedly stimulated by anisomycin or by sorbitol which was not changed by the co-expression of MKK3-AL, MKK3-WT, MKK6-AL, or MKK6-WT. GST/p38<sub>hog</sub> or GST/SAPK were affinity purified on glutathione beads and enzymatic activity was evaluated by in vitro kinase reactions. In Fig. 6A and B, graphical representation of p38<sub>hog</sub> and SAPK activity were markedly stimulated by anisomycin or by sorbitol which was not changed by the co-expression of MKK3-AL, MKK3-WT, MKK6-AL, or MKK6-WT. GST/p38<sub>hog</sub> or GST/SAPK were affinity purified on glutathione beads and enzymatic activity was evaluated by in vitro kinase reactions. In Fig. 6A and B, graphical representation of p38<sub>hog</sub> and SAPK activity were markedly stimulated by anisomycin or by sorbitol which was not changed by the co-expression of MKK3-AL, MKK3-WT, MKK6-AL, or MKK6-WT. GST/p38<sub>hog</sub> or GST/SAPK were affinity purified on glutathione beads and enzymatic activity was evaluated by in vitro kinase reactions. In Fig. 6A and B, graphical representation of p38<sub>hog</sub> and SAPK activity were markedly stimulated by anisomycin or by sorbitol which was not changed by the co-expression of MKK3-AL, MKK3-WT, MKK6-AL, or MKK6-WT. GST/p38<sub>hog</sub> or GST/SAPK were affinity purified on glutathione beads and enzymatic activity was evaluated by in vitro kinase reactions.
Fig. 7. MKK6-AL expression in COS cells prevents activation of p38\(\text{hog}\) after hyperosmolar stress but does not affect SAPK activation. pEBG/SAPK or pEBG/p38\(\text{hog}\) were transiently transfected into COS cells with pMT3/MKK6-AL, pMT3/MKK3-WT, or the empty pMT3 vector and stimulated with sorbitol for 30 min. Control cell samples, transfected with empty pMT3 and either pEBG/SAPK or pEBG/p38\(\text{hog}\), were left unstimulated. GST/p38\(\text{hog}\) or GST/SAPK were purified on glutathione-conjugated Sepharose. In vitro kinase reactions were performed to assess activity for each immunoprecipitate using the substrates myelin basic protein for p38\(\text{hog}\) and GST-Jun for SAPK. The results were analyzed by acrylamide gel electrophoresis followed by autoradiography. Densitometry values for each gel are graphically represented in each panel. GST/p38\(\text{hog}\) stimulation by sorbitol was inhibited by coexpression of MKK6-AL (A) while SAPK stimulation was unaffected (B).

Fig. 8. Association of eukaryotic MAP kinases with MAPK kinases in yeast. Fusion DNA constructs of pAS1 GAL4 DNA-binding domains and mammalian p38\(\text{hog}\) or SAPK were individually transfected into HF7c yeast with pACTII GAL4 DNA transactivating domain fusion constructs of MKK3, MKK6, or SEK-1. Yeast were selected on tryptophan/leucine-deficient medium, and expression of each fusion partner in individual yeast clones was confirmed by Western analysis as described. A, yeast colonies were plated uniformly on double minus medium and grown for 48 h. B, filter lift assay for \(\beta\)-galactosidase production demonstrating the association of SAPK with SEK-1 and the association of p38\(\text{hog}\) with all three MAPK kinases.

DISCUSSION

Lower eukaryotic cells have MAPK related signaling cascades which control diverse response to extracellular stimulation. In the budding yeast S. cerevisiae at least 5 physiologically distinct MAPK-related signaling pathways are known to exist (25) including two with homologues found in mammalian cells: the Fus3/Kss1 pathway which controls the mating response to extracellular pheromones (26, 27), and the HOG-1 pathway which results in the synthesis of intracellular glycerol to adapt to hyperosmolar stress (1). These kinases have highly similar sequences and are part of homologous activation pathways. To segregate components of distinct signaling cascades, members must have strict specificity for one another or alternatively, be physically compartmentalized. In the yeast mating factor cascade, Ste5 functions to bind together and compartmentalize three protein kinase components, Ste11, Ste7, and Fus3/Kss1 which have sequence similarity to mammalian MEKK, MEK, and MAPK, respectively (26, 28). Ste5 may therefore act as an insulator for this pathway by chaperoning these proteins and preventing association with other transduction cascades. Since no Ste5 homologues have been found in mammalian cells to date, similar signaling specificity might be provided by highly specific direct interactions between the mammalian protein kinases such as we have observed.

In mammalian cells three highly homologous MAPK signal transduction pathways have been identified, the ERK mitogenic cascade and the 2 stress activated cascades resulting in SAPK or p38\(\text{hog}\) activation. These kinase pathways fulfill distinct roles within cells and must be individually insulated or integrated in a coordinated way to maintain the specificity and diversity of extracellular signals. Since cells contain these and hundreds of other protein kinases which are often structurally highly related, there must be mechanisms to allow specificity. Here, we provide evidence for direct in vivo protein-protein interactions occurring between cognate components of the two most closely related of the three known mammalian MAPK related pathways, the SAPK and the p38\(\text{hog}\) pathways. The importance of these interactions in defining specificity is illustrated by the lack of specificity between the enzymes when mixed independently in vitro (22).

We have demonstrated that SAPK and p38\(\text{hog}\) are activated with both quantitative and qualitative differences after a variety of stress stimuli in both Jurkat lymphocytes and in NIH 3T3 cells. Such differential activation must reflect a divergence in activating pathways immediately upstream of these kinases. We show here that SEK1, a kinase which physiologically activates and associates with SAPK, is unable to bind to p38\(\text{hog}\) in vivo suggesting its independence from p38\(\text{hog}\) activation. MEKK, a kinase upstream of SEK1, while able to activate SAPK, is unable to activate p38\(\text{hog}\) even when overexpressed, suggesting a divergence of the SAPK and p38\(\text{hog}\) pathways at the level of MEKK. Furthermore, SEK-AL, a dominantly acting inhibitor of SAPK, while efficiently inhibiting SAPK activation, is unable to affect p38\(\text{hog}\) activation after hyperosmolar stress in COS cells. These observations imply that p38\(\text{hog}\) is not activated by SEK1 after stress stimuli in vivo, but becomes activated by a distinct kinase acting immediately upstream. MKK3 and MKK6 have been identified as mammalian homologues of SEK1 and likely induce activation of MAPK family kinases in response to extracellular stimuli (18–20). Both molecules can activate p38\(\text{hog}\) in vitro suggesting possible physiologic function. We have demonstrated that dominantly acting inhibitors of MKK3 and MKK6, MKK3-AL, and MKK6-AL respectively, prevent p38\(\text{hog}\) activation in response to hyperosmolar stress in COS cells but have no effect on SAPK activation. This is consistent with a role for each of these molecules as specific p38\(\text{hog}\) activators in vivo and demonstrates that the SAPK and the p38\(\text{hog}\) signaling pathways may be segregated in cells by specific intramolecular associations which have not been observed in vitro testing.

Although one S. cerevisiae MAPK signaling pathway is integrated through the assembly of individual components on scaffold proteins, to date proteins with similar function have not been identified in eukaryotic cells. We have demonstrated coimmunoprecipitation of members of cognate eukaryotic MAPK
signaling pathways from intact cells. These observations do not exclude the involvement of co-precipitating eukaryotic scaffolding proteins which may specifically assemble components of individual MAPK pathways. The yeast 2-hybrid assay has been used to demonstrate specific interaction between eukaryotic proteins when expressed as fusions with DNA transcription factor functional domains. To address the potential role of eukaryotic scaffolding proteins, individual MAPKs were expressed as GAL4 transcription factor DNA-binding domain fusions while their activators were expressed as GAL4 trans-activating fusions. Our data demonstrate that interaction occurs between the MAPK kinases and activator molecules in yeast without the participation of additional eukaryotic molecules. These observations, while not excluding the substitution of endogenous yeast molecules for eukaryotic scaffolding proteins, makes the participation of such molecules in eukaryotic MAPK signaling pathways less likely.

Why are two highly related stress kinases, triggered by similar agonists, activated through seemingly distinct mechanisms? The physiologic consequence of SAPK activation may be quite distinct from that of p38\(^{hog}\). MEKK induction in fibroblasts results in growth arrest suggesting that SAPK may lie on a growth inhibitory pathway, triggered by cellular stress and damage (21). This pathway may have evolved to allow damaged cells to repair themselves prior to division or to induce apoptotic death in severely damaged cells as suggested by recent work in NIH 3T3 and REF52 fibroblasts (29). Apoptosis as a consequence of SAPK activation has been directly demonstrated in the neuroectodermal cell line PC12 (30). After serum withdrawal, these cells, when differentiated by nerve growth factor (21), demonstrate an apoptosis-like death in response to heat shock and to the cytotoxic chemotherapeutic drug cis-platinum (31) which also is consistent with SAPK's role as a mediator of programmed cell death. While the consequences of specific activation of p38\(^{hog}\) are unknown, this protein kinase is specifically inhibited by pyridinyl-imidazole compounds, which act as powerful anti-inflammatory agents in vivo (32). p38\(^{hog}\) activation may therefore generate protective mechanisms in response to cellular stresses. This is consistent with data showing that p38\(^{hog}\), as an activator MAP kinase-associated protein kinase II (MAPKKAP kinase II), phosphorylates, and activates small molecular weight heat shock proteins providing protection from heat shock (33–36). A subtle balance may thus be struck between repair and growth arrest after stress stimuli that may be mediated differentially through each of these two pathways.

In vitro promiscuity of protein kinases can lead to artifactual connections between signaling elements. We have shown independence between SAPK and p38\(^{hog}\) phosphorylation implying a different physiologic role for each kinase and predict further downstream divergence of these pathways.

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