occurred in apes and Old World monkeys early in primate evolution, resulting in the haptoglobin gene, the haptoglobin-related gene, and the primate haptoglobin gene (*20*). In humans, a subsequent homologous unequal crossover took place, leaving the original haptoglobin gene and producing the human haptoglobin-related gene, a hybrid of the haptoglobin-related gene and the primate haptoglobin gene. In nonhuman primates, the sequence of the haptoglobin-related protein is known only for chimpanzees, where a frameshift leads to premature termination of translation (*20*). The absence of intact haptoglobin-related protein in chimpanzees is consistent with their lack of TLF activity and may explain why the primate most related to humans does not have this protective mechanism (*19*).

We hypothesize that the haptoglobin-related protein in TLF binds hemoglobin and is endocytosed by the trypanosome, where it is targeted to the lysosome. The low pH of the lysosome stimulates peroxidase activity of the complex, which then reacts with $H_2O_2$ to cause lipid peroxidation of the lysosomal membrane. The disrupted lysosome then releases its enzymes, and the trypanosome is autodigested.

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# Identification of a Dual Specificity Kinase That Activates the Jun Kinases and p38-Mpk2


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**One Ras-dependent protein kinase cascade leading from growth factor receptors to the ERK (extracellular signal–regulated kinases) subgroup of mitogen-activated protein kinases (MAPKs) is dependent on the protein kinase Raf-1, which activates the MEK (MAPK or ERK kinase) dual specificity kinases. A second protein kinase cascade leading to activation of the Jun kinases (JNKs) is dependent on MEKK (MEK kinase). A dual-specificity kinase that activates JNK, named JNKK, was identified that functions between MEKK and JNK. JNKK activated the JNKs but did not activate the ERKs and was unresponsive to Raf-1 in transfected HeLa cells. JNKK also activated another MAPK, p38 (Mpk2; the mammalian homolog of HOG1 from yeast), whose activity is regulated similarly to that of the JNKs.**


Three distinct protein kinase cascades activate members of the MAPK group in yeast (*1*). One such cascade exists in vertebrates in which Raf activates the MEK group of MAPK kinases (MAPKKs) (*2*), which in turn activate the ERK group of MAPKs (*3*). Raf, however, shares little similarity with the yeast MAPKK kinases (MAPKKKs) STE11 and BCK1, whose mammalian homolog is MEKK (*4*). Several mammalian MAPKs, the JNKs (*5*) or stress-activated protein kinases (*6*) and p38 or Mpk2 (*7*), that are similarly activated by a wide range of physiological and stressful stimuli (*5–9*), have been molecularly cloned. Although MEKK can activate MEK (*4, 10*), it is a more efficient activator of the JNK cascade, mediating responses to oncogenic Ras and growth factors (*11*). We have now identified a JNK-activating protein kinase, JNKK, that acts between MEKK and JNK. JNKK also activates p38, thus explaining the similarity between its response to extracellular stimuli to that of JNK. JNKK, however, does not activate the ERKs and is unresponsive to Raf-1. Hence, MEKK and JNKK form a protein kinase cascade that leads to JNK activation. This cascade, which stimulates c-Jun transcriptional activity, is distinct from the one involving Raf, MEK, and ERK.


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Both JNK1 and p38 complement an osmosensitive yeast mutant defective in the MAPK homolog, HOG1 (*7, 8*), suggesting that a mammalian homolog of PBS2, the MAPKK that activates HOG1 (*12*) and can also activate JNK1 (*8*), is their physiologic activator. Because MEK1 and MEK2 do not activate JNK (*11*), we searched for new mammalian MAPKKs that exhibit this function. Vertebrate complementary DNA (cDNA) clones encoding potential MAPKKs—XMEK2 from *Xenopus* and its mouse homolog, MMA1-SEK1—have been isolated (*13*). We isolated and determined the sequence of their human homolog (*14*) (Fig. 1A), and a clone encoding a truncated version of this protein was isolated by Dérijard *et al.* (*15*). The three vertebrate MAPKK-like proteins are very similar to each other (>95% amino acid identity within the kinase domains) and are more similar to PBS2 than to other *Saccharomyces cerevisiae* MAPKK homologs (*16*). Both the mouse and human MAPKK-like proteins can partially complement a PBS2 deficiency (Fig. 1B) (*17*). Transformation with vectors encoding the mammalian MAPKK sequences allowed *pbs2*$^-$ cells to grow in the presence of 0.9 M NaCl, albeit somewhat more slowly than *PBS2*$^+$ cells. Neither clone complemented a HOG1 deficiency or a double deficiency in MKK1 and MKK2 (*17*).

MMA1-SEK1 cDNA was expressed in *Escherichia coli* as a glutathione S-transferase (GST) fusion protein (*18*), purified, and examined for its ability to activate GST-JNK1 in a coupled kinase assay using GST–c-Jun(1–79) (*19*) as a substrate (Fig. 2A). GST-JNK1 alone exhibited little activity,

but after incubation with GST-MMA1 in the presence of adenosine triphosphate (ATP), its c-Jun kinase activity was stimulated manyfold. Because of its ability to activate JNK, we refer to MMA1-SEK1 as mJNKK. Similar results have been reported (*13*), and similar activity was exhibited by the human homolog, hJNKK (*20*). Catalytically inactive JNKK, in which lysine 116 was replaced by an arginine JNKK(K116R) (*21*), did not stimulate JNK1 activity (Fig. 2A). JNKK did not phosphorylate c-Jun directly (Fig. 2A) and did not phosphorylate ERK2 or stimulate its activity either in vitro or in vivo (*22*). ERK2, however, was fully activated by MEK1 and MEK2 (*22*). In addition to JNK1, JNKK efficiently phosphorylated and activated p38 that was expressed in *E. coli*. as a GST fusion protein (Fig. 2B). Catalytically inactive JNKK(K116R) did not phosphorylate or activate p38.

Activation of JNK requires phosphorylation at $Thr^{183}$ and $Tyr^{185}$ (*5*). We subjected $^{32}P$-labeled JNKK-phosphorylated JNK1 to phosphopeptide mapping and phosphoaminoacid analyses. Most of the phosphate incorporated into JNK1 was in a single peptide that comigrated with I1, one of the two isomeric phosphopeptides that appear after in vivo activation of JNK1 (Fig. 3A). JNKK-phosphorylated JNK1 contained phosphotyrosine (P-Tyr) and phosphothreonine (P-Thr) and phosphoserine (P-Ser) at a ratio of 7:1:1 (Fig. 3B). Although phosphopeptide I1 contained mostly P-Tyr, I2 contained both P-Tyr and P-Thr (Fig. 3B). The greater relative amount of P-Tyr in JNKK-phosphorylated JNK1 may result from preferred phosphorylation of $Tyr^{185}$, which also occurs in vivo after ultraviolet (UV) irradiation (Fig. 3B) or T cell activation (*23*). Similar results were obtained with JNKK-phosphorylated p38 (Fig. 3C). Activation of JNK1 by JNKK apparently requires Thr phosphorylation, because JNK1 was inactivated by protein phosphatase 2A, which removes phosphates from P-Thr (and P-Ser) but not P-Tyr (*22*). JNKK did not activate the T183A and Y185F mutants (*5*) of JNK1 (*22*). Therefore, like the MEKs, JNKK is a dual-specificity kinase.

We examined whether JNKK activates JNK and p38 in vivo. HeLa cells were transfected with expression vectors encoding epitope-tagged JNK1 (*11*) and mJNKK (*18*). After 48 hours, HA-JNK1 was isolated and

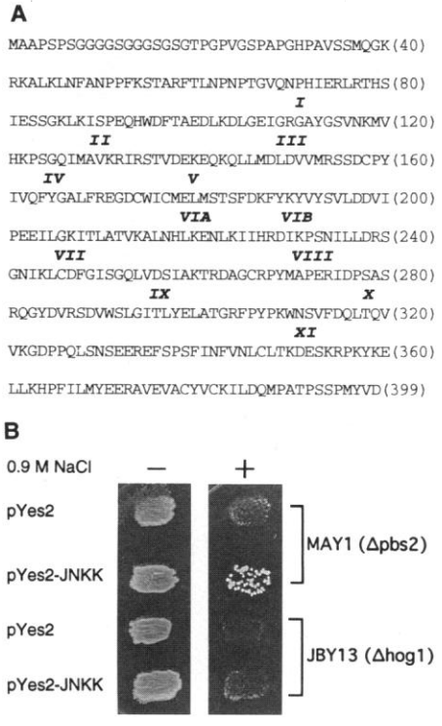


**Fig. 1.** Amino acid sequence of hJNKK and complementation of PBS2 deficiency in yeast. (**A**) The amino acid sequence of human JNKK. The protein kinase subdomains are denoted by roman numerals. (**B**) Complementation of a *pbs2*$^-$ yeast strain by JNKK. A DNA fragment encoding mJNKK(MMA1-SEK1) was inserted into the yeast expression vector pYES2 (Invitrogen) and transformed into the indicated yeast strains (*17*). Transformants were selected and replica patched on YPD plates without or with 0.9 M NaCl (*17*). After 4 days at 30°C the plates were photographed.

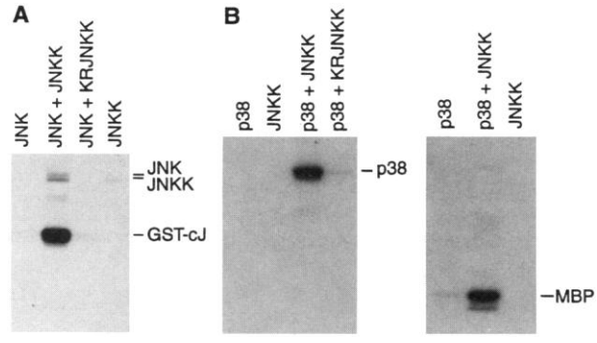


**Fig. 2.** Activation of JNK1 and p38 by JNKK. (**A**) Coupled JNKK assay. Purified GST-JNK1 was incubated at 30°C for 30 min in JNKK buffer (*30*) containing ATP (100 μM) without or with purified GST-mJNKK or catalytically inactive GST-mJNKK(K116R) (KRJNKK), as indicated (0.5 μg each protein). GST-mJNKK alone was incubated with ATP in JNKK buffer. The proteins were isolated on GSH-agarose and, after washing, incubated with GST-cJun(1–79) in JNK buffer (*18*) and [$\gamma$-$^{32}P$]ATP (10 μM) for 30 min at 30°C. Phosphorylated proteins were separated by SDS–polyacrylamide gel electrophoresis (PAGE) and visualized by autoradiography. (**B**) Phosphorylation and activation of p38. Purified GST-p38 (provided by R. Davis) was incubated for 30 min at 30°C in JNKK buffer with 10 μM [$\gamma$-$^{32}P$]ATP in the absence or presence of GST-mJNKK or GST-mJNKK(K116R), as indicated (0.5 μg each protein). The phosphorylated proteins were analyzed by SDS-PAGE and autoradiography (left panel). GST-p38 and GST-mJNKK (0.5 μg each) were incubated for 30 min at 30°C in JNKK buffer containing 100 μM ATP, either alone or in combination. The proteins were isolated on GSH-agarose, washed, and assayed for p38 activity with MBP (2 μg) as a substrate (right panel).

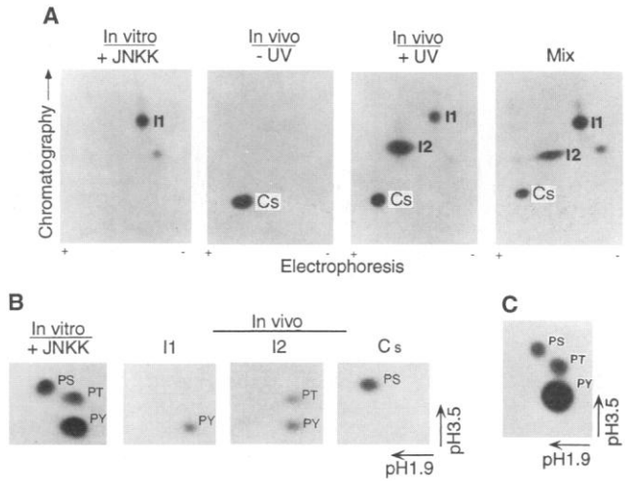


**Fig. 3.** Phosphopeptide mapping and phosphoaminoacid analysis of JNK1 and p38. (**A**) Phosphopeptide maps of GST-JNK1 phosphorylated in vitro by GST-JNKK and HA-JNK1 labeled in vivo. GST-JNK1 was phosphorylated by GST-JNKK as described (Fig. 2A), except we used 10 μM [$\gamma$-$^{32}P$]ATP for 1 hour. After SDS-PAGE, the GST-JNK1 band was excised and subjected to phosphopeptide mapping as described (*5, 31*). HA-JNK1 was transiently expressed in either nonirradiated or UV-irradiated HeLa cells that were incubated with $^{32}P$; HA-JNK1 was isolated as described (*5, 11*) and subjected to phosphopeptide mapping. I1 and I2 refer to the major phosphopeptides that appear after UV irradiation, containing one or two phosphates on the same peptide, respectively. Cs refers to a constitutively labeled phosphopeptide. The tryptic phosphopeptide maps of endogenous JNK1 isolated with a monoclonal antibody from the same cells were identical (*22*). (**B**) Phosphoaminoacid analysis. GST-JNK1 phosphorylated by GST-JNKK was subjected to phosphoaminoacid analysis (*31*). The I1, I2, and Cs phosphopeptides of in vivo labeled HA-JNK1 were extracted from the thin layer plate, and subjected to phosphoaminoacid analysis. $^{32}P$-labeled phosphoaminoacids were detected by autoradiography (20 hours at −70°C) and quantitated with a phosphorimager; nonradioactive standards were detected by ninhydrin staining. PS, PT, and PY: P-Ser, P-Thr, and P-Tyr, respectively. (**C**) GST-p38 was phosphorylated by GST-JNKK using [$\gamma$-$^{32}P$]ATP as described above and subjected to phosphoamino-acid analysis.

its c-Jun kinase activity was examined. Depending on the amount of the JNKK expression vector transfected, JNK1 was activated up to 128-fold (Fig. 4A). JNKK is likely to be activated through phosphorylation by a MAPKKK. MEKK is a MAPKKK that activates the JNK pathway much more efficiently than it does the ERK pathway (*11*). In a cotransfection assay in which a suboptimal amount of MEKK gave only 4-fold activation of JNK1 we observed up to 10-fold potentiation of JNK1 activation by JNKK (Fig. 4A). Similar results were obtained with expression vectors encoding full-length hJNKK and a truncated version initiated at $Met^{37}$ (hJNKKΔ), transfected either into HeLa or COS-1 cells (Fig. 4, B and D). These results, however, do not rule out the possibility that MEKK and JNKK act at different levels of two separate, but parallel, pathways leading to JNK activation. Therefore, we examined whether catalytically inactive JNKK(K116R) could inhibit JNK activation by MEKK. Indeed, a dose-dependent decrease in JNK1 activation by MEKK was observed upon cotransfection of HA-JNK1 with the JNKK(K116R) expression vector (Fig. 4C). This inhibition did not result from decreased expression or recovery of HA-JNK1. MEKK acts downstream of Ha-Ras in the signaling pathway leading to JNK activation (*11*). Cotransfection with JNKK(K116R) inhibited the modest activation of JNK1 by Ha-Ras (Fig. 4C). This inhibition was not the result of an adverse effect on HA-JNK1 expression. The same experiments were repeated with an expression vector encoding epitope-tagged p38 (*15*). As p38 is a poor c-Jun kinase, we measured its activity in an in-gel kinase assay with myelin basic protein (MBP) as a substrate (*24*). Like JNK1, p38 activity was stimulated up to 10-fold by cotransfection of the hJNKK vector (1 μg). However, unlike JNK1, p38 activity was only weakly potentiated by the cotransfected MEKK vector (in the presence or absence of the JNKK vector) in either COS-1 (Fig. 4D) or HeLa cells (*22*).

We transfected a mammalian GST-JNKK expression vector into HeLa cells in the absence or presence of an MEKK expression vector. Cells transfected with the GST-JNKK vector alone were either incubated with epidermal growth factor (EGF) or left untreated. GST-JNKK was isolated and tested for activation of recombinant JNK1. GST-JNKK isolated from untreated cells stimulated JNK1 activity by fourfold, whereas GST-JNKK isolated from EGF-treated cells was threefold more active (Fig. 5A). Coexpression with MEKK resulted in at least a 12.5-fold increase in JNKK activity, yielding 52-fold activation of JNK1. The increase in GST-JNKK activity was not the result of an increase in expression or recovery of the enzyme (*25*).

We incubated recombinant GST-JNKK with extracts of either unstimulated or EGF-stimulated HeLa cells, or HeLa cells transfected with either JNKK or MEKK expression vectors. Extracts of MEKK-transfected cells phosphorylated recombinant GST-JNKK 18-fold more efficiently than extracts of either unstimulated or JNKK transfected

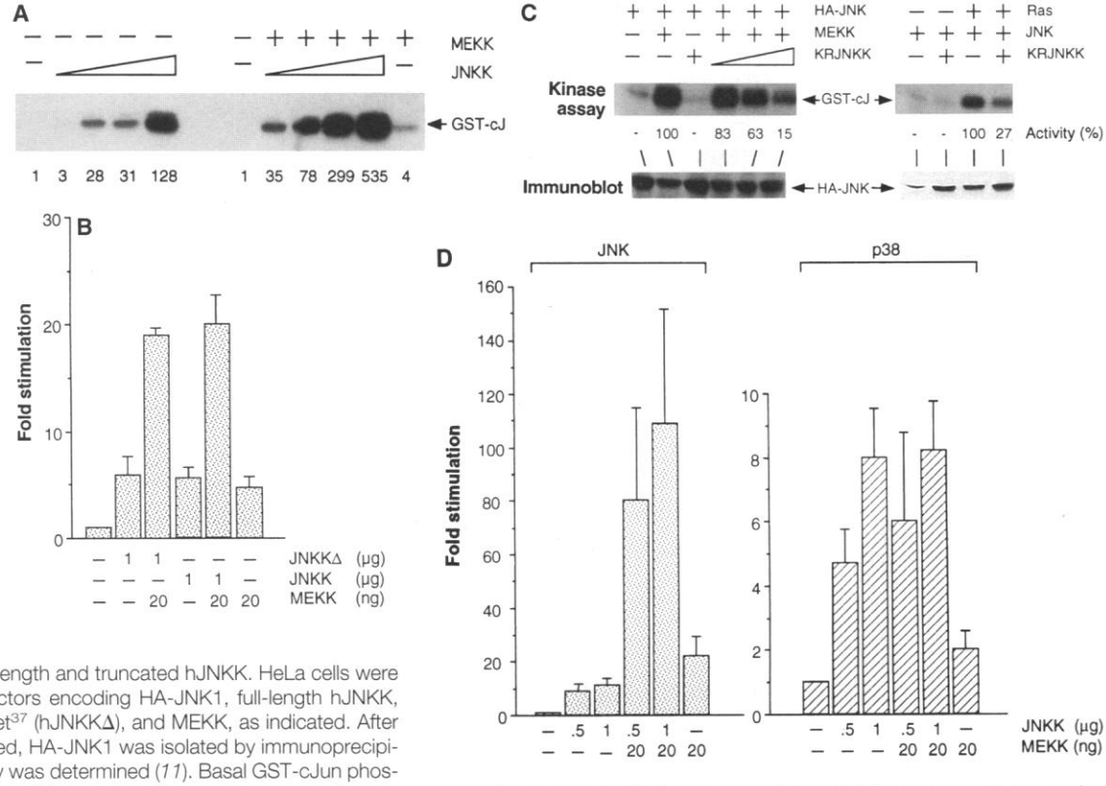


**Fig. 4.** Activation of JNK1 and p38 by JNKK in vivo. (**A**) Synergistic activation of JNK1 by cotransfected JNKK and MEKK expression vectors. HeLa cells were transiently transfected with pSRα-HA-JNK1 (1 μg per plate) in the absence or presence of pSRα-mJNKK (0.2, 0.5, 1.0, and 2.0 μg) and pCMV5-MEKK (20 ng per plate) expression vectors, as indicated. DNA concentration was kept constant by supplementation with pSRα. After 48 hours, the cells were collected, HA-JNK1 was immunoprecipitated, and its activity was determined as described (*11*). Substrate phosphorylation was quantitated with a phosphorimager. Basal GST-cJun phosphorylation by JNK1 alone was 113 counts per minute (cpm). Fold stimulation above that activity is indicated. (**B**) Comparison of full-length and truncated hJNKK. HeLa cells were transfected with expression vectors encoding HA-JNK1, full-length hJNKK, truncated hJNKK initiated at $Met^{37}$ (hJNKKΔ), and MEKK, as indicated. After 48 hours, the cells were collected, HA-JNK1 was isolated by immunoprecipitation, and its Jun kinase activity was determined (*11*). Basal GST-cJun phosphorylation by HA-JNK1 alone was 13 cpm. Fold stimulation above that level is indicated. The averages of two experiments (means ± SD) are shown. (**C**) Inhibition of MEKK- or Ha-Ras–induced JNK1 activation by catalytically inactive JNKK. HeLa cells were transfected with HA-JNK1 (1 μg per plate), MEKK (0.2 μg per plate), Ha-Ras($Leu^{61}$) (1.0 μg per plate) or JNKK(K116R) (0.2, 0.5, or 1.0 μg; + refers to 1.0 μg per plate) expression vectors, as indicated. DNA concentrations were kept constant with pSRα. After 48 hours, the cells were collected and HA-JNK1 activity was determined. A sample of each lysate was analyzed for its content of HA-JNK1 by immunoblotting (*11*). Substrate phosphorylation was quantitated with a phosphorimager and the maximal activities obtained after stimulation with MEKK (left panel) or Ha-Ras (right panel) were given values of 100% (corresponding to 2107 and 104 cpm, respectively). (**D**) Activation of p38 and JNK1 by JNKK. COS-1 cells were transfected with expression vectors encoding HA-JNK1 (1.0 μg per plate), Flag-p38 (0.1 to 1.0 μg per plate), hJNKK, or MEKK, as indicated. After 48 hours the cells were collected and HA-JNK1 or Flag-p38 was isolated by immunoprecipitation. HA-JNK1 activity was determined as described above, whereas Flag-p38 activity was determined by an in-gel kinase assay with MBP as a substrate (*24*). Substrate phosphorylation was quantitated with a phosphorimager and fold-stimulation of HA-JNK1 and Flag-p38 kinase activities were calculated. The results shown are averages (mean ± SD) of three separate experiments.

cells, whereas extracts of EGF-stimulated cells were 3.4-fold more active than the control extracts (Fig. 5B). An extract of HeLa cells transfected with a truncated form of MEKK (MEKKΔ; *11*) also phosphorylated GST-JNKK(K116R) and stimulated GST-JNKK activity 22-fold, whereas an extract of cells transfected with catalytically inactive MEKK(KM) (*11*) failed to do so (Fig. 5C). MEKKΔ immunopurified from transiently transfected COS-1 cells efficiently phosphorylated GST-JNKK(K116R), whereas immune complexes isolated from COS-1 cells transfected with the empty expression vector did not (Fig. 5C). JNKK was also

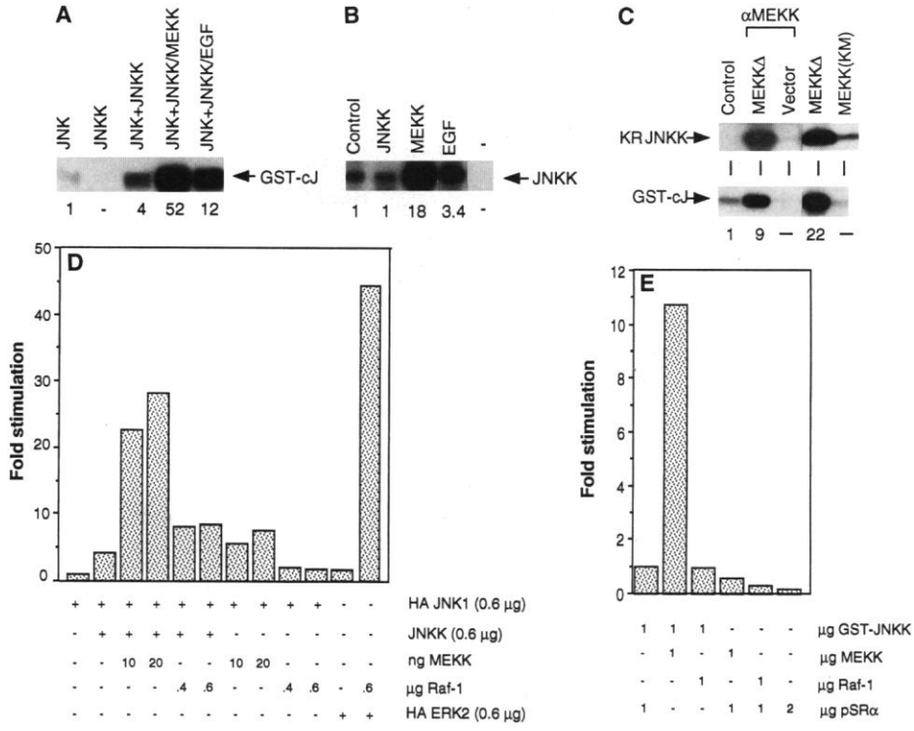


**Fig. 5.** Activation and phosphorylation of JNKK by MEKK. (**A**) Activation of JNKK by cotransfected MEKK. HeLa cells were transfected with 1 μg per plate of either pCMV5 (lane 1) or GST-mJNKK mammalian expression vector (lanes 2 to 5) without (lanes 1 to 3 and 5) or with pCMV5-MEKK (0.5 μg per plate; lane 4). After 48 hours the cells were either treated with EGF (20 ng/ml; lane 5) for 15 min or left untreated (lanes 1 to 4) and extracts were prepared. GST-JNKK was isolated on GSH-agarose beads and assayed for activation of GST-JNK1 as described (Fig. 2A). In lane 2 no GST-JNK1 was added. Fold stimulation above the activity of JNK1 incubated with mock-transfected cell extract (lane 1; 123 cpm) is indicated below each lane. (**B**) Phosphorylation of GST-JNKK by transfected cell extracts. HeLa cells were transiently transfected with GST-JNKK (lane 2), MEKK (lane 3), or empty expression vectors (lanes 1, 4, and 5). After 48 hours the cells transfected with the empty vector were incubated without (lanes 1 and 5) or with EGF (lane 4) for 15 min before harvesting. Recombinant GST-JNKK (0.5 μg) was incubated for 1 hour in JNKK buffer with ATP, isolated on GSH-agarose beads, and incubated with 30-μg samples of the various extracts at 30°C for 30 min in JNKK buffer with 10 μM [$\gamma$-$^{32}$P]ATP (except lane 5 in which GSH-agarose beads without GST-JNKK were incubated with control extract). After extensive washing, phosphorylated proteins were analyzed by SDS-PAGE and autoradiography. Fold increase above the amount of JNKK phosphorylation by the control cell lysate (lane 1; 340 cpm) is indicated. (**C**) Activation and phosphorylation of JNKK by immunopurified MEKK. COS-1 cells were transfected with pCMV5-MEKKΔ or pCMV5 (1 μg; lanes 2 and 3). After 48 hours the cells were lysed and MEKKΔ was immunoprecipitated (*10*). HeLa cells were transfected with 0.5 μg of MEKKΔ, MEKK(K432M) (lanes 4 and 5), or empty expression vectors (lane 1) and extracted after 48 hours. The immunecomplexes and HeLa cell extracts were assayed for their ability to phosphorylate recombinant GST-JNKK-(K116R) (upper panel) and stimulate GST-JNKK activity (lower panel) in a coupled kinase assay, as described above. Fold stimulation of JNKK activity (basal activity; 1075 cpm) is indicated. (**D**) MEKK, but not Raf-1, potentiated JNK activation by JNKK. HeLa cells were transiently cotransfected with HA-JNK1, HA-ERK2, JNKK, MEKK, or activated Raf-1 expression vectors, as indicated. After 48 hours the cells were harvested and HA-JNK1 and HA-ERK2 were immunoprecipitated and their kinase activities were determined as described (*11*). Fold stimulations above their basal activities were calculated and plotted. (**E**) Selective activation of JNKK. HeLa cells were transiently transfected with GST-JNKK, MEKK, activated Raf-1, or empty expression vectors, as indicated. After 48 hours cells were harvested and GST-JNKK was isolated and its ability to activate GST-JNK1 in a coupled kinase assay was determined as described in (A). Fold stimulation above its basal activity was calculated and plotted.

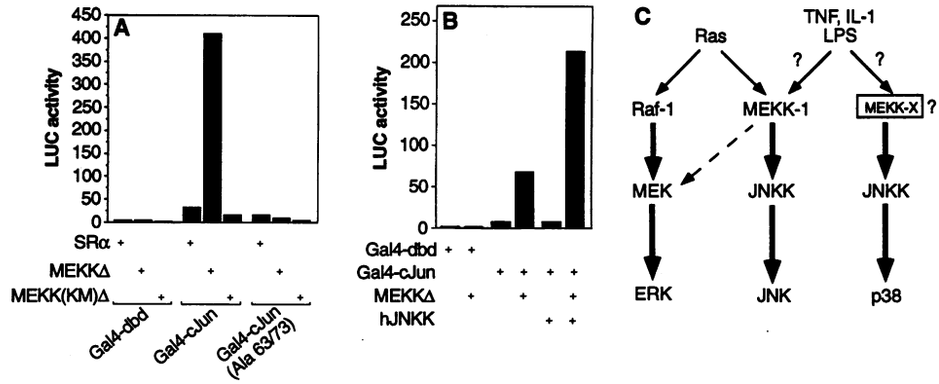


**Fig. 6.** Stimulation of c-Jun transcriptional activity and summary. (**A**) CV-1 cells were cotransfected with a 5xGAL4-Luc reporter plasmid (1 μg per plate) and expression vectors (10 ng each) for GAL4 DNA binding domain (dbd), GAL4-cJun(1–223), GAL4-cJun(1–223; Ala63/73), MEKKΔ, and catalytically inactive MEKK(KM)Δ, as indicated [see (*9*) and (*11*) for description of reporter and expression plasmids]. After 20 to 24 hours the cells were collected, and luciferase activity was determined and normalized to the protein content of each extract. Shown are the averages of two experiments. Luciferase activity expressed by cells transfected with GAL4-dbd and pSRα was given an arbitrary value of 1. (**B**) CV-1 cells were transfected with the 5xGAL4-Luc reporter and GAL4-dbd, GAL4-cJun(1–223), MEKKΔ, and hJNKK expression vectors (10 ng each), as indicated. After 24 hours the cells were collected and relative luciferase activity was determined and calculated as described above. (**C**) Summary figure illustrating the organization of the three known vertebrate MAPK cascades leading to activation of ERK, JNK, and p38. The thick solid arrows denote the major activation cascades and the broken arrow illustrates the potential activation of MEK by large amounts of MEKK (shown as MEKK-1). The putative MEKK-like enzyme that leads to p38 activation is denoted as MEKK-X. Thin arrows stand for inputs from upstream signals (Ras, tumor necrosis factor, interleukin-1, and lipopolysaccharide) into the three kinase cascades. The steps that have not yet been tested are indicated by question marks.

phosphorylated by immunopurified intact MEKK, but not MEKK(KM) (*26*). In a coupled kinase assay containing immune complexes, MEKKΔ or MEKK stimulated the activity of bacterially expressed GST-JNKK in activating JNK1 by ninefold (Fig. 5C) (*26*).

In comparison to MEKK, Raf-1 was an inefficient activator of JNKK. In transient cotransfections, activated Raf-1 had a marginal effect on JNK1 activation, although it stimulated ERK2 activity by 10-fold (Fig. 5D). Suboptimal doses of MEKK, on the other hand, potentiated JNKK activity 7- to 10-fold (Figs. 4B and 5D). In a coupled kinase assay activated Raf-1 did not stimulate the activity of GST-JNKK (Fig. 5E), whereas MEKK stimulated its activity up to 12.5-fold (Figs. 3B and 5E). Raf-1 and MEKK had no effect on expression of GST-JNKK or HA-JNK1 (*25*).

We examined the involvement of MEKK and JNKK in regulation of c-Jun transcriptional activity, which is stimulated in response to its phosphorylation at $Ser^{63}$ and $Ser^{73}$ (*27*). The transcriptional activity of a fusion protein consisting of the GAL4 DNA binding domain and the c-Jun transactivation domain (GAL4-cJun) (*9*), measured by a reporter plasmid containing a luciferase gene driven by a GAL4 responsive promoter, was stimulated 13-fold in cells cotransfected with an MEKKΔ expression vector (Fig. 6A). An expression vector encoding catalytically inactive MEKKΔ did not stimulate GAL4-cJun activity, neither did active MEKKΔ potentiate the activity of a GAL4-cJun fusion protein containing alanines instead of serines at positions 63 and 73 of the c-Jun activation domain. A suboptimal amount of MEKKΔ expression vector by itself potentiated GAL4-cJun activity by 9-fold; cotransfection with a small amount of a JNKK expression vector potentiated this effect, leading to 30-fold activation (Fig. 6B).

Our results suggest that MEKK phosphorylates and activates JNKK which in turn phosphorylates and activates the JNKs. The JNKs phosphorylate c-Jun and potentiate its transcriptional activity. In addition to the JNKs, JNKK also phosphorylates and activates p38. Although MEKK potentiates the ability of JNKK to activate JNK in three different cell types (HeLa, COS-1, and PC12) (*22*), it had only a weak effect on p38 activity in either the presence or absence of cotransfected JNKK. In vitro, however, extracts of MEKKΔ-transfected cells activated p38 nearly as efficiently as they activated JNK1 (*28*). A possible explanation for this puzzling result is the existence of adaptor proteins, which function similarly to STE5 in yeast (*29*). By forming a complex with STE11, STE7, and FUS3, STE5 increases both the efficiency and specificity of signal transmission within and by that cascade (*29*). One STE5-like protein might mediate the formation of a MEKK:JNKK:JNK complex, whereas another might promote formation of a complex encompassing p38, JNKK, and a yet-to-be described MEKK-like kinase. The relative expression levels of these proteins might determine which MAPK is most effectively stimulated by a given extracellular stimulus. Clearly the protein kinase cascade composed of Raf-1, MEK, and ERK is distinct from the cascades leading to JNK or p38 activation (Fig. 6C).

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14. The human (h) JNKK cDNA clone was isolated by screening a λZAPII human T lymphocyte Jurkat cDNA library (Stratagene) with an MMA1-SEK1 cDNA (provided by L. Zon) as a probe. One nearly full-length clone was obtained after screening $5 \times 10^5$ phage. Reverse transcriptase–mediated polymerase chain reaction (RT-PCR) was used to complete the entire coding region with primers: 5′-TGGGCAATCACTACTCCGCATTACTAC-3′, 5′-CTGCCATTATTTGCCCACTTGGTTTGTGG-3′, and an anchor primer 5′-CTGGTTCGGCCCACCTCTGAAGGTTCCAGAATCGATAG-3′. The nucleotide sequence of hJNKK was determined on both strands by the dideoxy chain termination method using Sequenase Version II (U.S. Biochemical Corp.) and a model 373A automated sequencer (Applied Biosystems). Accession number for the JNKK sequence is U177433.
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16. The catalytic domain of hJNKK is 48%, 41%, and 37%, identical to the catalytic domains of PBS2, MKK1, and STE7, respectively.
17. Expression vectors encoding yeast, human, and mouse (m) JNKK were constructed by subcloning a Bam H1 fragment encoding mJNKK and a Bam HI–Xba I fragment encoding hJNKK into the plasmid pYes2 (Invitrogen) and transformed into various yeast strains, MAY1 (*pbs2*Δ), JBY13 (*hog1*Δ), and 3233-1B (*mkk1*Δ,*mkk2*Δ), by standard methods. The osmotic stress assay was done as described (*12*). Whereas cells transformed with mJNKK and hJNKK grew on 0.9 M NaCl plates after 4 days, *PBS2*$^+$ cells grew within 1 to 2 days. The complementation of mutants in cell wall biosynthesis was done as described [K. Ire *et al.*, *Mol. Cell. Biol.* **13**, 3076 (1993)]. The 3233-1B cells transformed with mJNKK grew only after 7 or 8 days on 1% glucose at 37°C, whereas wild-type cells grew within 1 to 2 days.
18. To construct GST-JNK1 an Nco 1–Sal 1 fragment encoding JNK1, pSRα-HA-JNK1 (*11*) was subcloned into pGEX-KG, a GST-fusion protein expression vector (*3*); GST-JNKK bacterial expression vectors were constructed by inserting either a Bam HI fragment encoding mJNKK or PCR-generated Bam HI–Pst I and Pst I–Bam HI fragments, which together regenerated the entire hJNKK sequence, into pGEX-KG. The fusion proteins were expressed in *E. coli* and purified as described (*18*). The mammalian GST-JNKK expression vector, pEBG-GST-JNKK, was constructed by first subcloning a Bgl II–Bam H1 fragment encoding GST, derived from pGEX-2T (Pharmacia, Inc.), into pEBB [S. Mizushima and S. Nagata, *Nucleic Acids Res.* **18**, 5322 (1990)]. Next a Bam H1 fragment encoding mJNKK was subcloned into the resulting vector. A Bam H1 fragment encoding mJNKK was subcloned into the Bgl II site of pSRα to generate an mJNKK expression vector. The mJNKK(K116R) expression vector was similarly constructed. The hJNKK expression vectors were constructed by inserting two PCR-generated Bam HI fragments encoding either the full-length protein or a truncated version initiated at Met37 (hJNKKΔ) into the Bgl II site of pSRα.
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23. B. Su and M. Karin, unpublished data.
24. Due to coprecipitation of an unrelated MBP kinase activity it was necessary to measure p38 activity by an in-gel kinase assay (*5, 18*). Immunecomplexes of transiently expressed Flag-p38 were separated on SDS-polyacrylamide gels containing MBP (0.5 mg/ml). After electrophoresis the proteins were renatured and kinase activity was determined by incubation in kinase buffer containing [$\gamma$-$^{32}$P]ATP. By performing a similar analysis of JNK1 activity we determined that the in-gel kinase assay produces results that are essentially identical to immunecomplex kinase assays.
25. A. Minden and A. Lin, unpublished data. Expression of HA-JNK1 was examined as described with antibody to the HA epitope (*11*), expression of GST-JNKK was examined by immunoblotting with an antibody to GST and expression of Flag-p38 (*15*) was detected with an antibody to the Flag epitope (*5*).
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33. Phosphatase buffer contains 20 mM Hepes (pH 7.0), 10 mM $MgCl_2$, 1 mM $MnCl_2$, 1 mM DTT, 100 μg/ml BSA, 50 μM leupeptin, 10 μg/ml aprotinin, 10 μg/ml pepstatin A (Calbiochem).


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