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Mammalian c-Jun N-terminal kinase pathway and STE20-related kinases

Review Article

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Summary

The c-Jun N-terminal kinases (JNKs) belong to a subgroup of mitogen-activated protein kinases (MAPKs) that are activated by environmental stress, proinflammatory cytokines, and mitogenic stimuli in mammalian cells. Studies on the JNK pathway in mammalian cells demonstrate that JNK regulates the transcriptional activities of many transcription factors, and that JNK is required for the regulation of cell proliferation and apoptosis. Studies on *jnk*-deficient mice reveal that JNK is involved in the response to immunological stimuli and in embryonic morphogenesis. JNK, as other MAPKs, is regulated by a kinase cascade. JNK activation is mediated by dual phosphorylation on the motif, Thr-Pro-Tyr. To date, this phosphorylation is known to be mediated by the MAPK kinases (MAP2Ks), MKK4 and MKK7. MKK4 and MKK7 are activated by MEKK1 and other MAPK kinase kinases (MAP3Ks). The MAPK kinase kinase kinases (MAP4Ks) including HPK1, GCK, and homologous kinases, which have a kinase domain related to yeast STE20, can activate the JNK signaling cascade. These mammalian STE20-related MAP4Ks may be involved in integrating various stimuli to the JNK cascade. The signaling specificity of mammalian JNK pathway may be controlled by scaffold proteins that interact with kinases at different levels in the pathway.

I. Introduction

Mitogen-activated protein kinases (MAPKs) are important mediators for intracellular signaling in cells (Schaeffer and Weber, 1999). Mammalian MAPKs consist of three major groups including extracellular signal-regulated kinases (ERKs), c-Jun N-terminal kinases (JNKs, also known as stress-activated protein kinases, SAPKs), and p38-MAPKs (Schaeffer and Weber, 1999). All of MAPKs share a common character: they are activated by phosphorylation at a Thr-X-Tyr motif (X is Glu in ERKs, Pro in JNKs, and Gly in p38-MAPKs) in kinase subdomain VIII (Schaeffer and Weber, 1999). The major targets for MAPK kinases are transcription factors that regulate gene expression. MAPKs are involved in signaling induced by various extracellular or intracellular stimuli. Currently, the JNK pathway is one of the known cellular signaling pathways that respond to the most diverse stimuli (Ip and Davis, 1998; Schaeffer and Weber, 1999). JNK is activated by mitogenic signals such as epidermal growth factor (Minden *et al.*, 1994b), lymphocyte activation signals (Su *et al.*, 1994; Sakata *et al.*, 1995; Berberich *et al.*, 1996; Chen *et al.*, 1996a; Chen *et al.*, 1996b), and oncogenic Ras (Derijard *et al.*, 1994). JNK is also activated by pro-inflammatory cytokines (TNF-α and IL-1) (Kyriakis *et al.*, 1994; Sluss *et al.*, 1994), lipopolysaccharide (Hambleton *et al.*, 1996), G protein-coupled receptors (Collins *et al.*, 1996; Coso *et al.*, 1996), shear stress (Li *et al.*, 1996), osmotic shock (Galcheva-Gargova *et al.*, 1994), protein synthesis inhibitors (Kyriakis *et al.*, 1994), and apoptotic stimuli such as growth factor withdrawal (Xia *et al.*, 1995), heat shock (Kyriakis *et al.*, 1994; Zanke *et al.*, 1996), ceramides (Westwick *et al.*, 1995), DNA-damaging chemicals (Saleem *et al.*, 1995; Zanke *et al.*, 1996), UV radiation (Derijard *et al.*, 1994; Chen *et al.*, 1996b; Zanke *et al.*, 1996), and γ radiation (Kharbanda *et al.*, 1995; Chen *et al.*, 1996a; Chen *et al.*, 1996b). The diversity of JNK-activating stimuli imply that mammalian cells may be equipped with multiple upstream regulators that link various cellular signals to the JNK pathway, and the accumulated experimental evidence proves that is the case. To date, the JNK pathway consists of JNKs and various MAP2Ks, MAP3Ks, and MAP4Ks (**Figure 1**). The subtle regulation of

the JNK pathway by its regulators in conjunction with other signaling pathways may allow JNK to regulate a variety of cellular functions. In this review, we will discuss the known components in the JNK pathway and how the emerging mammalian scaffold proteins may control signaling diversity and specificity in this signaling pathway.

II. c-Jun N-terminal kinases

The human JNKs are encoded by three genes *jnk1*, *jnk2*, and *jnk3* (Derijard *et al.*, 1994; Kallunki *et al.*, 1994; Sluss *et al.*, 1994; Gupta *et al.*, 1996). The corresponding genes have also been identified in rats (Kyriakis *et al.*, 1994). JNK3 is preferentially expressed in neuronal tissues, while JNK1 and JNK2 are widely expressed in many tissues. Ten isoforms of JNK, generated by alternative splicing of the transcripts from the three genes, have been identified (Gupta *et al.*, 1996). The protein products of the JNK isoforms have molecular weights of 46 kDa or 55 kDa. The 55 kDa JNK isoforms contain a C-terminal extension, a result of alternative splicing, which distinguishes them from the 46 kDa isoforms (Gupta *et al.*, 1996). No apparent functional differences exist among the 46 kDa and 55 kDa isoforms encoded by the same JNK gene (Gupta *et al.*, 1996). An additional alternative splicing exists in the kinase domains of JNK1 and JNK2, but not in JNK3 (Gupta *et al.*, 1996). The alternative splicing in the kinase domains of JNK1 and JNK2 changes the specificity of interaction between JNKs and their substrates (Gupta *et al.*, 1996), suggesting that JNK isoforms may target different substrates *in vivo*. The JNK binding sites are different from the sites of phosphorylation on the substrates (Kallunki *et al.*, 1996). Deletion of the binding site prevents phosphorylation of the substrate by JNK (Kallunki *et al.*, 1996). However, a substrate lacking a JNK-binding site can also be phosphorylated through association with a protein containing the JNK-binding region (Kallunki *et al.*, 1996).

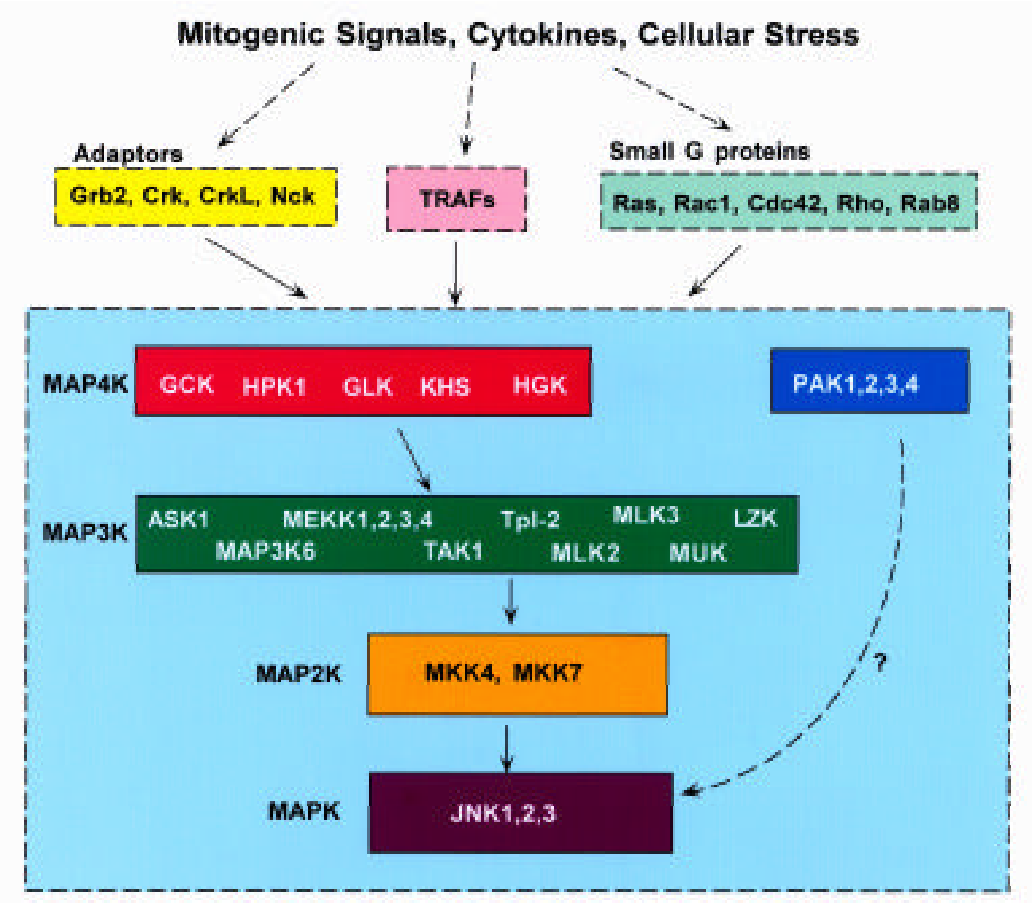

Figure 1. The mammalian JNK signaling pathway. Currently known MAPKs, MAP2Ks, MAP3Ks, and MAP4Ks in the JNK pathway are illustrated schematically. PAKs are capable of activating JNK; however, the direct link between PAKs and the JNK signaling module has not been established. The activation of the JNK pathway is known to be mediated by adaptor molecules, p21 small G proteins, or TNF-receptor-associated factors (TRAFs). The signaling specificity among the components is not presented in this figure.

The known substrates for JNK family members include the transcription factors c-Jun (Hibi *et al.*, 1993; Derijard *et al.*, 1994; Kyriakis *et al.*, 1994; Gupta *et al.*, 1996), JunD (Gupta *et al.*, 1996), ATF-2 (Gupta *et al.*, 1995; van

Dam *et al.*, 1995; Whitmarsh *et al.*, 1995), ATFa (Bocco *et al.*, 1996), Elk-1 (Cavigelli *et al.*, 1995; Whitmarsh *et al.*, 1995; Zinck *et al.*, 1995), Sap-1a (Janknecht and Hunter, 1997), GABPα, GABPβ (Hoffmeyer *et al.*, 1998), and the tumor suppressor p53 (Milne *et al.*, 1995; Alder *et al.*, 1997). Generally, phosphorylation of these factors by JNK increases their transcriptional activity.

The physiological functions of JNK have been examined by genetic analysis. The *jnk1*$^{-/-}$, *jnk2*$^{-/-}$, and *jnk3*$^{-/-}$ single mutant mice have no global abnormality (Yang *et al.*, 1997b; Dong *et al.*, 1998; Yang *et al.*, 1998). The T cells in *jnk1*$^{-/-}$ and *jnk2*$^{-/-}$ mice preferentially differentiate into Th2 rather than Th1 cells (Dong *et al.*, 1998; Yang *et al.*, 1998). The *jnk1*$^{-/-}$ T cells also hyper-proliferate and exhibit decreased activation-induced apoptosis (Dong *et al.*, 1998). Excitotoxicity-induced apoptosis in the hippocampus is absent in *jnk3*$^{-/-}$ mice in comparison to normal mice (Yang *et al.*, 1997b). The *jnk1/jnk3* and *jnk2/jnk3* deficient mice also develop normally (Kuan *et al.*, 1999); however, *jnk1/jnk2* deficient mice are embryonically lethal and have severe dysregulation of apoptosis in the brain (Kuan *et al.*, 1999). These results indicate that JNK1 and JNK2 may have overlapping functions, and are important in regulation of immune response and embryonic development. JNK3 may have its unique functions in the neuronal tissues. These studies also provide animal models which support the accumulated evidence on the role of JNK in apoptotic signaling in mammalian cells (Ip and Davis, 1998).

III. MAP2Ks in the JNK pathway

The activation of JNK is dependent on the phosphorylation on Thr-183 and Tyr-185. MKK4 (also known as SEK1 or JNKK1) is a physiological activator of JNK (Sanchez *et al.*, 1994; Derijard *et al.*, 1995; Lin *et al.*, 1995). MKK4 phosphorylates and activates JNK *in vitro* and *in vivo* (Sanchez *et al.*, 1994; Derijard *et al.*, 1995; Lin *et al.*, 1995). However, recombinant wild-type JNK proteins are phosphorylated at Tyr, Ser and Thr residues in the presence of recombinant MKK4, whereas a kinase-inactive JNK is phosphorylated predominantly on Tyr (Sanchez *et al.*, 1994). This suggests that recombinant MKK4 does not have apparent dual specificity toward JNK. It is possible that the phosphorylation on Thr-183 is caused by the proline-directed kinase activity of JNK itself, occurring after MKK4-mediated Tyr-phosphorylation. Another possibility is that MKK4 obtains dual-specific kinase activity only after activation by upstream kinases. Two isoforms of MKK4 have been reported through the differential usage of translation initiation sites (Derijard *et al.*, 1995; Lin *et al.*, 1995).

MKK4 has been found to be mutated or deleted in some tumor cells, suggesting that it may be a tumor suppressor gene (Teng *et al.*, 1997; Su *et al.*, 1998). Homologous deletion in *mkk4* genes is embryonically lethal in mice, indicating that MKK4 is essential for embryonic development (Nishina *et al.*, 1997; Yang *et al.*, 1997a). Studies in *mkk4*$^{-/-}$/*rag*$^{-/-}$ chimaeric mice reveal that MKK4 protects thymocytes from apoptosis mediated by CD95 and CD3 (Nishina *et al.*, 1997), and is required for maintenance of a normal peripheral lymphoid compartment but not for lymphocyte development (Swat *et al.*, 1998). *Mkk4*$^{-/-}$ T cells derived from *mkk4*$^{-/-}$/*rag*$^{-/-}$ chimaeric mice are defective in heat shock and anisomycin-induced JNK activation, but normal in osmotic shock-induced JNK activation (Nishina *et al.*, 1997). These results indicate that MKK4 is one but not the only activator of JNK in mammalian cells.

Recently, a novel kinase MKK7 (also named as JNKK2) has been cloned and found to specifically activate JNK, but not p38-MAPK or ERK (Moriguchi *et al.*, 1997; Tournier *et al.*, 1997; Wu *et al.*, 1997; Yao *et al.*, 1997). MKK7 is related to MKK4 and belongs to the mammalian MAPK kinase superfamily (Tournier *et al.*, 1997; Yao *et al.*, 1997). MKK7 is also closely related to the *Drosophila* protein kinase hemipterous (HEP) (Tournier *et al.*, 1997; Yao *et al.*, 1997), which is the activator of *Drosophila* JNK (DJNK).

Both MKK4 and MKK7 are widely expressed in human and murine tissue, whereas the relative abundance of each MKK differs among tissues (Tournier *et al.*, 1997; Yao *et al.*, 1997). Both MKK4 and MKK7 mediate signals from the same panel of extracellular stimuli (Wu *et al.*, 1997); however, studies show that they are preferentially activated by different MAP3Ks (Hirai *et al.*, 1998; Merritt *et al.*, 1999; Tournier *et al.*, 1999). Furthermore, the MKK7 gene can encode six isoforms of protein products through alternative splicing of the mRNA transcripts (Tournier *et al.*, 1999). These MKK7 isoforms respond differently to extracellular stimuli and upstream kinases (Tournier *et al.*, 1999). The differential regulation of MKK4 and MKK7 isoforms by their upstream activators needs to be further examined.

IV. MAP3Ks in the JNK pathway

Multiple upstream MAPK/ERK kinase kinases or MAP kinase kinase kinases (MEKK or MAP3Ks) have been reported to activate the JNK pathway via MKK4 and/or MKK7 (**Figure 1**). These MEKK-like kinases include MEKK1-4, ASK1/MAPKKK5, MAPKKK6, TAK1, Tpl-2/Cot, MLK2/MST, MLK3/SPRK/PTK1, MUK/DLK/ZPK, and LZK.

A. MEKKs

MEKK1 is the first identified MAP3K that activates JNK (Minden *et al.*, 1994a; Yan *et al.*, 1994). MEKK1 was cloned on the basis of its homology with the yeast STE11 and Byr2 kinases (Lange-Carter *et al.*, 1993; Xu *et al.*, 1996). To date, four kinases have been cloned and named MEKK1-4 (Lange-

Carter *et al.*, 1993; Blank *et al.*, 1996; Gajewski and Thompson, 1996; Xu *et al.*, 1996; Ellinger-Ziegelbauer *et al.*, 1997; Gerwins *et al.*, 1997; Takekawa *et al.*, 1997). The four MEKKs (ranging from 69.5-195 kDa in size) have homologous kinase domains in the C-termini of the proteins; however, their N-terminal domains have little homology. MEKK1 and MEKK4 can interact with GTP-binding proteins Cdc42 and Rac (Fanger *et al.*, 1997; Gerwins *et al.*, 1997). MEKK1 also binds to Ras in a GTP-dependent manner (Russell *et al.*, 1995). All four MEKKs (MEKK1-4) activate the JNK pathway (Lange-Carter *et al.*, 1993; Blank *et al.*, 1996; Gajewski and Thompson, 1996; Xu *et al.*, 1996; Ellinger-Ziegelbauer *et al.*, 1997; Gerwins *et al.*, 1997; Takekawa *et al.*, 1997). Besides the JNK pathway, MEKKs also regulate other cellular signaling pathways. MEKK1, MEKK2, and MEKK3 activate the ERK pathway (Lange-Carter *et al.*, 1993; Blank *et al.*, 1996; Ellinger-Ziegelbauer *et al.*, 1997), and also activate the NF-κB through the IκB kinases (IKKs) (Lee *et al.*, 1997; Zhao and Lee, 1999). MEKK3 and MEKK4 have been shown to activate the p38-MAPK pathway through MKK6 (Takekawa *et al.*, 1997; Deacon and Blank, 1999).

B. TAK1

TGF-β activated kinase 1 (TAK1) was identified by its ability to rescue STE11 mutants in *S cerevisiae* (Yamaguchi *et al.*, 1995). TAK1 is a 579 amino acid protein with the kinase domain in its N-terminus (Yamaguchi *et al.*, 1995). The C-terminal region has no distinct domain structures but interacts with TAK1 binding protein (TAB) 1 and 2 (Yamaguchi *et al.*, 1995; Shibuya *et al.*, 1996). Association of TAK1 and TAB1 enhances the kinase activity of TAK1 (Shibuya *et al.*, 1996). TAK1 is activated by TGF-β (Yamaguchi *et al.*, 1995), interleukin 1 (Ninomiya-Tsuji *et al.*, 1999), ceramide, and UV-C treatments (Shirakable *et al.*, 1997). TAK1 activates JNK and p38, but has no effect on ERK (Yamaguchi *et al.*, 1995; Wang *et al.*, 1997). TAK1 also indirectly activates IKK activity and NF-κB transcriptional activity (Ninomiya-Tsuji *et al.*, 1999). *Xenopu*s TAK1 and TAB1 are important in the dorsoventral patterning of early embryos (Shibuya *et al.*, 1998). Ectopic expression of TAK1 induces apoptosis in early *Xenopu*s embryos (Shibuya *et al.*, 1998).

C. ASK1/MAPKKK5 and ASK2/MAPKKK6

Apoptosis signal-regulating kinase 1 (ASK1, also named MAPKKK5) was identified by a polymerase chain reaction (PCR)-based strategy (Wang *et al.*, 1996; Ichijo *et al.*, 1997). ASK1 consists of 1375 amino acids with a molecular weight about 155 kDa, and the kinase domain of ASK1 is in the middle part of the protein (Wang *et al.*, 1996; Ichijo *et al.*, 1997). ASK1 has been shown to activate JNK and p38-MAPK through MKK4 and MKK3 respectively (Wang *et al.*, 1996; Ichijo *et al.*, 1997). ASK1 is activated by TNF-α, and dominant-negative ASK1 suppresses TNF-α-induced apoptosis (Ichijo *et al.*, 1997). ASK1 is also activated by Daxx, a Fas-binding protein, and is activated by Fas ligation (Chang *et al.*, 1998). ASK1 has also been shown to be involved in apoptosis induced by oxidative stress (Gotoh and Cooper, 1998; Saitoh *et al.*, 1998), microtubule-interfering agents (Wang *et al.*, 1998a), and genotoxic chemicals (Chen *et al.*, 1999b). Overexpression of ASK1 is capable of inducing apoptosis in transfected cells (Ichijo *et al.*, 1997).

An ASK1-related kinase kinase, MAPKKK6 (murine homologue is ASK2), was identified by yeast two-hybrid screen using ASK1 as a bait (Wang *et al.*, 1998b). MAPKKK6 also interacts with ASK1 when coexpressed in 293 cells (Wang *et al.*, 1998b). Overall, MAPKKK6 is 45% homologous to ASK1, and the kinase domain of MAPKKK6 is 82% identical to that of ASK1. The catalytic domain of MAPKKK6 shares 37, 42, 43, and 42% identity to MEKK1, MEKK2, MEKK3, and MEKK4, respectively (Wang *et al.*, 1998b). In contrast to ASK1, which is a strong JNK and p38-MAPK activator, MAPKKK6 only weakly activates JNK1 but does not activate p38-MAPK or ERK (Wang *et al.*, 1998b).

D. Tpl-2/Cot

Tumor progression locus 2 (Tpl-2) was originally identified as a proto-oncogene that is involved in T lymphomas induced by Moloney murine leukemia virus (Patriotis *et al.*, 1993). Tpl-2 is about 90% identical to the human *cot* gene, which was first identified by its transforming ability (Miyoshi *et al.*, 1991). The Tpl-2 kinase domain shares approximately 30-35% identity to other JNK-activating MAP3Ks; however, the overall similarity between Tpl-2 and other MAP3Ks is low. Expression of Tpl-2 in mammalian cells activates ERK and JNK through the direct phosphorylation of MEK-1 and MKK4, respectively (Salmeron *et al.*, 1996). Tpl-2 has also been shown to participate in CD3 and CD28-induced NF-κB activation through NF-κB-inducing kinase (NIK) and the IKKs (Lin *et al.*, 1999). Tpl-2 also activates the nuclear factor of activated T cells (NF-AT) and induces IL-2 expression in T-cell lines (Tsatsanis *et al.*, 1998).

E. MLKs

The mixed lineage kinase (MLK) family is a group of kinases consisting of MLK1 (Dorow *et al.*, 1993), MLK2/mammalian STE20-like (MST) (Dorow *et al.*, 1993; Katoh *et al.*, 1995), MLK3/src-homology 3 (SH3) domain-containing proline-rich kinase (SPRK)/protein tyrosine kinase 1 (PTK-1) (Rena *et al.*, 1996; Teramoto *et al.*, 1996; Tibbles *et al.*, 1996), MAPK-upstream kinase (MUK)/dual leucine zipper-bearing kinase (DLK)/leucine-zipper protein kinase (ZPK) (Fan *et al.*, 1996; Hirai *et al.*, 1996), and leucine zipper-bearing kinase (LZK) (Sakuma *et al.*, 1997). This group of kinase is characterized by their catalytic domains which show structural features of both tyrosine- and

serine/threonine-specific protein kinase. MLKs contain an SH3 motif at the N-terminus and proline-rich regions at the C-terminus. MLKs also have Leu/Ile-zipper motifs near the C-terminus. These motifs may allow MLKs to dimerize or interact with other molecules. The effect of MLK1 on the MAPK pathways is unknown. MLK2/MST activates the JNK pathway through both MKK4 and MKK7 (Hirai *et al.*, 1998). However, MLK2/MST activates recombinant MKK7 more effectively than recombinant MKK4 (Hirai *et al.*, 1998). MLK2/MST weakly activates p38-MAPK and ERK (Hirai *et al.*, 1997). MLK3 activates the JNK and p38-MAPK pathway via MKK4 and MKK3/6, respectively, but has no effect on the ERK pathway (Tibbles *et al.*, 1996), MUK preferentially activates the JNK pathway (Hirai *et al.*, 1996), and utilizes MKK7 but not MKK4 as a substrate (Merritt *et al.*, 1999). LZK has been shown to activate JNK and induce c-Jun phosphorylation in transfected cells (Sakuma *et al.*, 1997), but its activity toward the other MAPK pathways is unclear. MLK1, 2, 3, and MUK contain potential binding sites (CRIB motifs) for Cdc42 and Rac. MLK2 and MLK3 have been shown to interact with Cdc42 and Rac proteins (Teramoto *et al.*, 1996; Nagata *et al.*, 1998).

V. STE20 related Kinases

The MAP kinase modules in *S. cerevisiae* are controlled by a MAP4K named STE20. Several kinases containing a kinase domain that is homologous to STE20 have recently been identified in mammalian cells (**Figures 2 and 3A**). A phylogenetic analysis on the protein sequences of these mammalian STE20-related kinases reveals that these kinases are divided into several subgroups (**Figure 4**), which roughly correspond to their structures and biochemical properties. The HPK1/GCK subgroup and HGK/NIK have been shown to regulate the JNK pathway through MAP3Ks. Therefore, they could be classified as MAP4Ks in mammalian cells.

A. PAK subgroup

p21-activated kinases (PAK1-4) are characterized by their ability to bind to the Ras-related small G-proteins, Rac1 and Cdc42, through their CRIB domains (Bagrodia *et al.*, 1995b; Martin *et al.*, 1995; Abo *et al.*, 1998) (**Figure 2**). The binding of PAKs to GTP-bound Rac1 or Cdc42 results in the autophosphorylation and activation of the kinase (Manser *et al.*, 1994; Bagrodia *et al.*, 1995b; Martin *et al.*, 1995; Teo *et al.*, 1995). Rac1 and Cdc42, as well as their direct guanine nucleotide exchange factors (GEF), Ost and Dbl, respectively, were shown to stimulate the JNK pathway via MEKK1 (Bagrodia *et al.*, 1995a; Coso *et al.*, 1995; Minden *et al.*, 1995; Olson *et al.*, 1995; Brown *et al.*, 1996). However, PAKs do not behave as MAP4Ks in the JNK pathway, since PAKs have no (Yablonski *et al.*, 1998; Zhou *et al.*, 1998) or only modest (Bagrodia *et al.*, 1995a; Polverino *et al.*, 1995; Frost *et al.*, 1996) effect on JNK activation. In addition, the direct interaction between PAKs and MAP3Ks has not been shown. It is found that PAK binding to Rac1 is dispensible for Rac1-induced activation of JNK1 (Westwick *et al.*, 1997). Since Cdc42 and Rac can directly interact with MEKK1, MEKK4, MLK2 and MLK3, PAKs may not be the direct link between small G proteins and the JNK kinase cascade. Neutrophil PAK1 and PAK2 have been shown to phosphorylate the $p47^{phox}$ subunit of NADPH oxidase in response to a chemotactic peptide fMetLeuPhe (Knaus *et al.*, 1995). Recombinant PAK also phosphorylate $p67^{phox}$ subunit of NADPH oxidase (Ahmed *et al.*, 1998). Therefore, it is possible that PAKs indirectly regulate JNK activation by inducing chronic oxidative stress through NADPH oxidase in cells.

B. GCK/HPK1 subgroup

The second subgroup of STE20-related kinases includes germinal center kinase (GCK), hematopoietic progenitor kinase 1 (HPK1), kinase homologous to STE20 (KHS)/GCK related kinase (GCKR), and GCK-like kinase (GLK). These kinases are around 96-97 kDa, and share a similar structural configuration. They have the STE20-like catalytic domain in their N-terminus, at least 2 proline-rich binding domains, which can bind to the Src homology 3 (SH3) domain, in their middle region. A domain distantly related to part of murine citron protein (citron homology domain, CNH domain) is located in the C-terminus of these kinases (**Figures 2 and 3**). This subgroup of STE20-related kinases have been shown to be strong and specific JNK activators.

Germinal center kinase (GCK) was first found to be expressed in B lymphocytes residing in the germinal center region of lymphoid follicles (Katz *et al.*, 1994), but later found to be ubiquitously expressed in many tissues. It is a potent and specific activator of JNK but not of ERK1 or p38-MAPK (Pombo *et al.*, 1995). GCK-mediated JNK activation was blocked by a dominant-negative MKK4/SEK construct which indicates that GCK also activates MKK4/SEK (Pombo *et al.*, 1995); however, a direct interaction between GCK and MKK4 has not been detected. GCK has been shown to interact with TNF receptor-associated factor-2 (TRAF2) and with MEKK1 (Yuasa *et al.*, 1998). Therefore, GCK may link the TNF receptor complex to the JNK pathway through MEKK1. GCK interacts with small G protein Rab8 (Ren *et al.*, 1996); however, the biological significance of this interaction is unclear.

Hematopoietic progenitor kinase 1 (HPK1) is preferentially expressed in hematopoietic cells, especially in lymphocytes (Hu *et al.*, 1996; Kiefer *et al.*, 1996). HPK1 does not contain CRIB motif and does not interact with Rac1 or Cdc42 (Hu *et al.*, 1996). HPK1 contains four proline-rich domains (putative SH3 domain-binding sites) (**Figure 2**). HPK1 has been shown

to interact with the adaptor molecules Grb2, Crk, CrkL and Nck (Anafi *et al.*, 1997; Oehrl *et al.*, 1998; Ling *et al.*, 1999). These adaptor proteins are involved in signaling induced by receptor-linked tyrosine kinases through their SH2 domains, which bind to phosphorylated tyrosine residues. Fas receptor signaling results in the caspase-mediated-cleavage of HPK1 at aspartic acid residue 385, which leads to an increase of HPK1 kinase activity and a decrease in its binding to Crk and Grb2 (Chen *et al.*, 1999a). Several pieces of evidence show that HPK1 is regulated by tyrosine kinases. HPK1 is activated by tyrosine phosphatase inhibitors and is tyrosine-phosphorylated after epidermal growth factor stimulation and T-cell receptor ligation (Anafi *et al.*, 1997; Ling *et al.*, 1999). The interaction between HPK1 and adaptor proteins may recruit HPK1 to surface receptor-tyrosine kinase complexes. HPK1 preferentially activates JNK but not ERK or p38-MAPK (Hu *et al.*, 1996; Kiefer *et al.*, 1996). HPK1 interacts with MEKK1 *in vivo* and directly phosphorylates its regulatory region *in vitro* (Hu *et al.*, 1996). HPK1 also interacts with MLK3 and TAK1 (Kiefer *et al.*, 1996; Zhou *et al.*, 1999). HPK1 is upstream of TAK1 in TGF-β induced JNK activation, and the interaction between HPK1 and TAK1 is enhanced by TGF-β treatment (Zhou *et al.*, 1999).

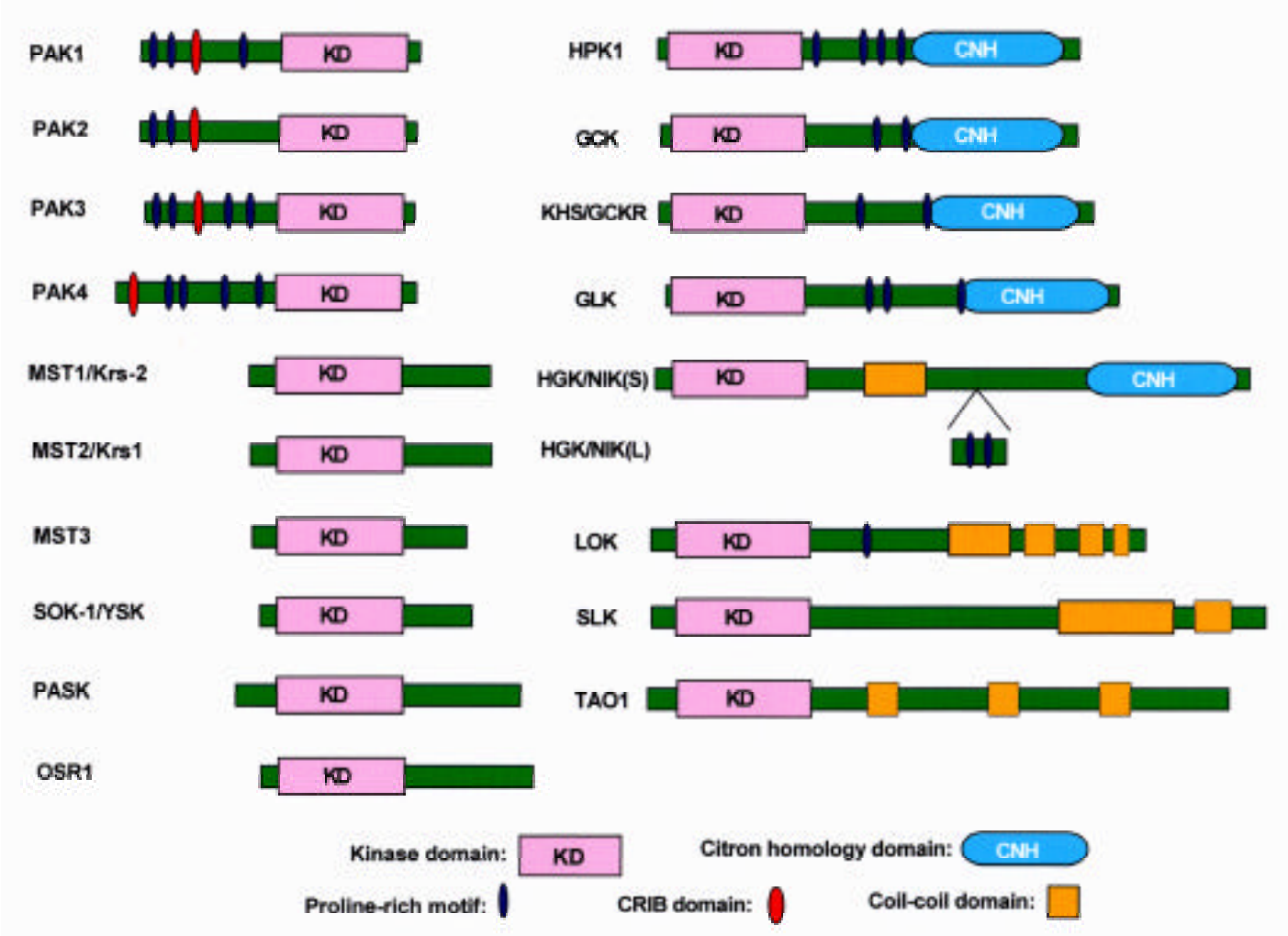

Figure 2. Structures of mammalian STE20-related kinases. The protein sequences of the STE20-related kinases were analyzed using the Web-based SMART program (simple modular architecture research tool, EMBL). Human kinase sequences are used in these analyses, except PASK (rat), SLK (murine), and TAO1 (rat).

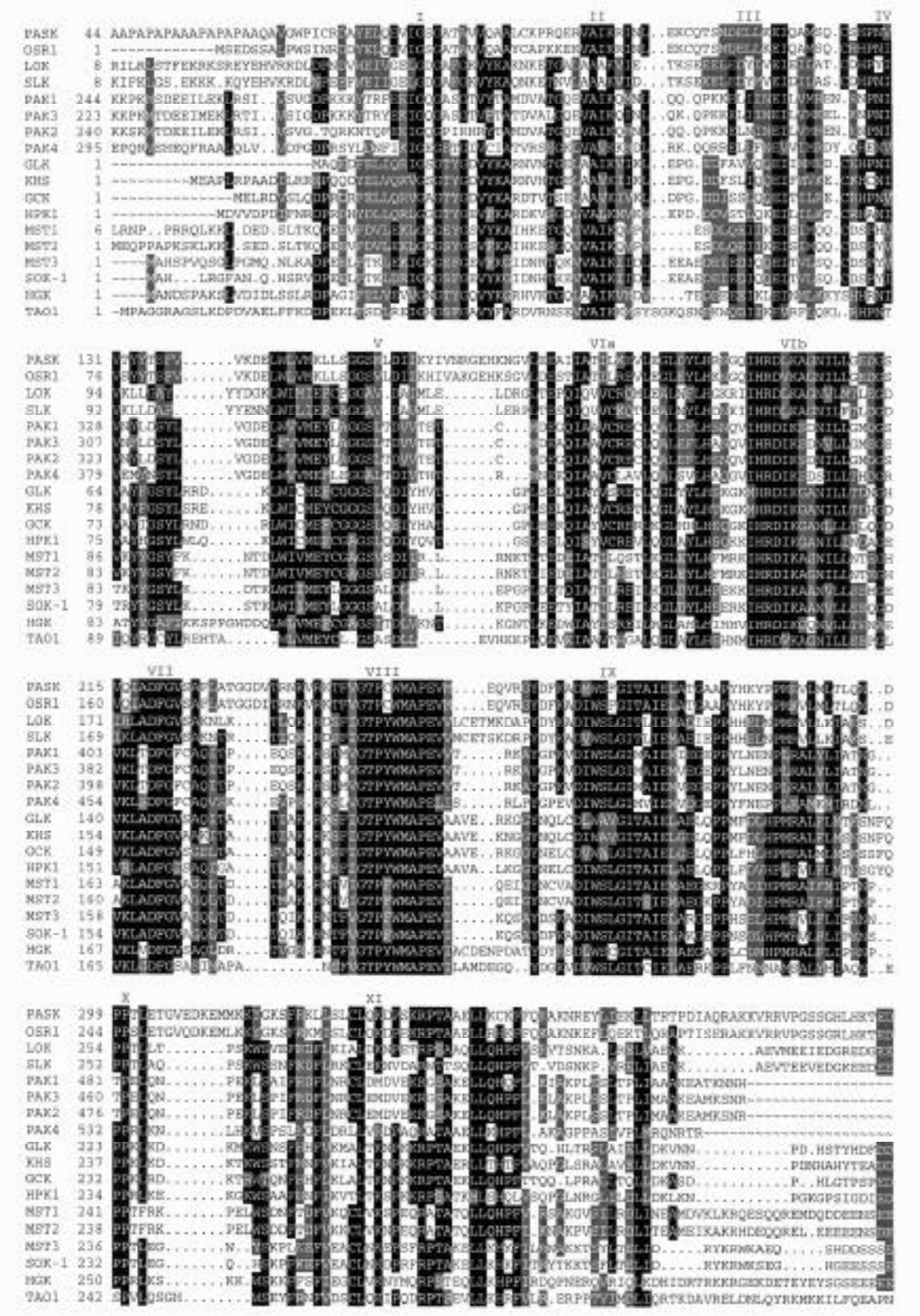

Figure 3. Sequence comparison among mammalian STE20-related kinases. (**A**) Alignment among the kinase domains of STE20-related kinases. The percentages of identity vary from 95% to 36%. Human kinase sequences are used in these analyses, except PASK (rat), SLK (murine), and TAO1 (rat).

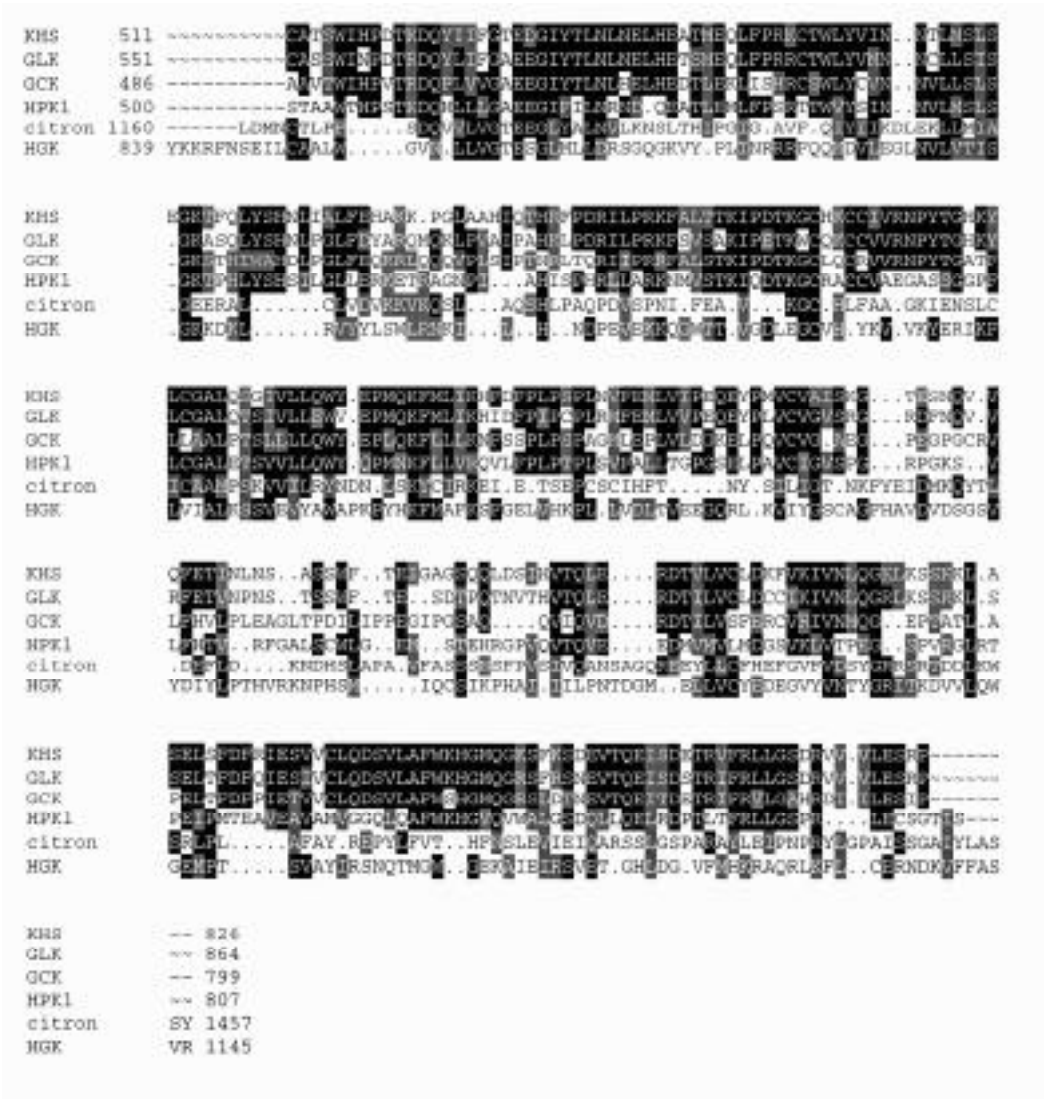

Figure 3 (Continued). (**B**) Alignment of citron homology domains of HPK1, GCK, KHS, GLK, HGK and murine citron protein. The sequences were analyzed using the PILEUP program (version 7.2; Wisconsin Genetics Computer Group). The output was processed using the BoxShade 3.21 program. Identical residues are shown in black shade, and conserved residues are shown in gray shade. Gaps introduced into the sequences to optimize the alignment are illustrated with dots.

KHS/GCKR is a kinase closely related to GCK and HPK1 (Shi and Kehrl, 1997; Tung and Blenis, 1997). KHS/GCKR also preferentially activates JNK but not p38-MAPK and ERK (Shi and Kehrl, 1997; Tung and Blenis, 1997). KHS/GCKR is activated by TNF-α and UV irradiation (Shi and Kehrl, 1997). A KHS/GCKR dominant-negative mutant or antisense suppresses TNF-α, TRAF2, and UV-induced JNK activation (Shi and Kehrl, 1997). KHS/GCKR also physically interacts with TRAF2 (Shi and Kehrl, 1997). KHS/GCKR interacts with the SH3 domains of Crk and CrkL, but not with the SH3 domains of Grb2 or Nck, through its proline rich domains (Oehrl *et al.*, 1998). KHS/GCKR is constitutively active in chronic myeloid leukemia (CML) cells and interacts with oncoprotein Bcr-Abl (Shi *et al.*, 1999). KHS/GCKR is activated by Bcr-Abl in a Ras-dependent manner (Shi *et al.*, 1999). A dominant-negative KHS/GCKR blocks Bcr-Abl-induced JNK activation (Shi *et al.*, 1999).

GLK is widely expressed in many tissues (Diener *et al.*, 1997). GLK preferentially activates JNK, but not p38-MAPK or ERK, when co-expressed in mammalian cells (Diener *et al.*, 1997). GLK-induced JNK activation is blocked by a dominant-negative mutant of MKK4 or MEKK1 (Diener *et al.*, 1997). GLK phosphorylates recombinant MEKK1 (Diener *et al.*, 1997). These data suggest that GLK regulates the JNK pathway through MEKK1 and MKK4. To date, GLK is known to be regulated by UV irradiation and TNF-α (Diener *et al.*, 1997). Since GLK also contains proline-rich motifs, like other kinases in this family (**Figure 2**), it may be regulated through interaction with SH3 domain-containing molecules.

C. *HGK, LOK, SLK, and TAO1*

Human HPK/GCK like kinase (HGK) is a 133.4 kDa protein (Yao *et al.*, 1999). Its murine counterpart, Nck-interacting kinase (NIK), is 98% identical to HGK, except for an insertion containing two proline-rich motifs in the middle region of the kinase (Su *et al.*, 1997) (**Figure 2**). A longer form of human HGK that contains these proline-rich motifs was also detected in brain tissue by RT-PCR (Yao *et al.*, 1999). However, the short form of HGK appears to be the predominant form in other human tissues including liver, skeletal muscle and placenta (Yao *et al.*, 1999). Murine HGK/NIK activates JNK and MKK4 when co-expressed in cells, and interacts with MEKK1 (Su *et al.*, 1997). Murine HGK/NIK strongly interacts with the SH3 domain of Nck, but not with other molecules that contain SH3 domains, such as Grb2 and phospholipase C-γ (Su *et al.*, 1997). Therefore, it may link Nck-mediated signals to the JNK pathway through MEKK1 and MKK4. Human HGK/NIK-induced JNK activation can be blocked by a dominant-negative mutant of MKK4, MKK7, or TAK1, but not by a dominant-negative MEKK1 mutant (Yao *et al.*, 1999). The difference between human and murine HGK/NIK (long versus short forms) is intriguing and needs to be further examined. Although HGK/NIK contains a citron-homology (CNH) domain (**Figure 2**), this domain shares low homology to the CNH domains in HPK1, GCK, KHS, and GLK (**Figure 3B**). HGK/NIK also contains a coiled-coil domain that is not found in any other kinase of the GCK/HPK1 subgroup (**Figure 2**). A phylogenetic analysis reveals that HGK does not belong to the HPK1/GCK subgroup (**Figure 4**).

Lymphocyte-oriented kinase (LOK) is a 130 kDa kinase which contains a STE20-like kinase domain in its N-terminus followed by a proline-rich region that is a potential SH3 domain binding site, and a long coiled-coil structure at its C-terminus (Kuramochi *et al.*, 1997; Kuramochi *et al.*, 1999) (**Figure 2**). LOK, unlike HPK1/GCK subgroup, only weakly activates the JNK and p38-MAPK pathways by itself (Kuramochi *et al.*, 1997). LOK may have a specific function in lymphocytes; however, the physiological stimuli and downstream effectors for LOK are unknown (Kuramochi *et al.*, 1997).

Thousand and one amino acid protein kinase 1 (TAO1) was identified by a PCR-based method from a rat brain cDNA library (Hutchison *et al.*, 1998). TAO1 is preferentially expressed in brain and testis (Hutchison *et al.*, 1998). TAO1 contains a kinase domain which shares 40-50 % homology with other STE20-related kinases, and a C-terminal regulatory domain which contains coiled-coil structures (Hutchison *et al.*, 1998) (**Figure 2**). TAO1 activates recombinant MKK4, MKK3, and MKK6 *in vitro*; however, it preferentially interacts with MKK3 and only activates MKK3 when co-expressed in mammalian cells (Hutchison *et al.*, 1998).

STE20-like kinase (SLK) is a 141 kDa kinase (Itoh *et al.*, 1997; Pytowski *et al.*, 1998). The N-terminal catalytic domain of SLK shares 70% identity with LOK's kinase domain, and 40-45% identity with the kinase domains of other STE20-related kinases. The C-terminus of SLK shares 40% homology with LOK, 20% homology with HGK and TAO1, and no homology with other STE20-related kinases. The specificity of SLK toward the known MAPK pathways is unknown.

D. MST/SOK subgroup

This subgroup includes mammalian STE20 like 1 (MST1)/kinase regulated by stress 2 (Krs2) (Creasy and Chernoff, 1995a; Taylor *et al.*, 1996), MST2/Krs1 (Creasy and Chernoff, 1995b; Taylor *et al.*, 1996), MST3 (Schinkmann and Blenis, 1997), STE20/oxidant stress response kinase-1 (SOK-1)/yeast Sps1/Ste20-related kinase 1 (YSK1) (Pombo *et al.*, 1996; Osada *et al.*, 1997), and proline-alanine-rich Ste20-related kinase (PASK) (Ushiro *et al.*, 1998). These kinases are approximately 45-55 kDa and share 55-90% identity in their STE20-like kinase domain. These kinases have not been shown to activate any of the MAPK pathways, however, their abilities to affect MAPK signaling in the presence of MAPK-inducing agents have not been tested. MST1/Krs2 and MST2/Krs1 are activated by extreme heat shock (55°C), arsenite, okadaic acid, and staurosporine (Taylor *et al.*, 1996). MST1/Krs2 is also activated by *in vitro* incubation with purified PP2A (Creasy and Chernoff, 1995a). SOK-1/YSK1, as its name indicates, is activated by oxidative stress such as H_2O_2 (Pombo *et al.*, 1996). The stimuli that activate MST3 are unknown.

VI. Scaffold Proteins

Since JNK is regulated by such diverse upstream pathways, the mechanism by which cells ensure signaling specificity is intriguing. In *S. Cerevisiae*, the MAPK kinase pathways are coordinated by scaffold proteins (Herskowitz, 1995; Whitmarsh and Davis, 1998). The Ste5p protein binds the components of the MAPK module that control mating (Herskowitz, 1995; Whitmarsh and Davis, 1998). Ste5p interacts with the MAP3K Ste11, the MAP2K Ste7, and the MAPKs Fus3p and Kss1p through different regions of the protein. Pbs2p is a scaffold protein that coordinates an osmoregulatory MAPK pathway (Herskowitz, 1995; Whitmarsh and Davis, 1998). In contrast with Ste5p, Pbs2p is not only a scaffold, but also a protein kinase component (MAP2K) of this MAPK pathway. In mammalian cells, the understanding of scaffold proteins is limited.

Recent studies indicate that mammalian cells do contain proteins which serve as scaffolds. One such protein which facilitates signaling of the JNK pathway has been identified. JNK-interacting protein 1 (JIP1) was isolated by a two-hybrid screen for proteins that bind to JNK. JIP1 preferentially binds to JNK, but not to ERK or p38-MAPK (Whitmarsh *et al.*,

1998). JIP1 was first characterized as a JNK inhibitor, because it inhibits the nuclear translocation of JNK and suppresses JNK-mediated functions including transformation and apoptosis (Dickens *et al.*, 1997). However, further characterization of JIP1 has shown that JIP1 interacts with multiple components of the JNK cascade (Whitmarsh *et al.*, 1998). JIP1 binds to MKK7 but not MKK4, which activates both JNK and p38-MAPK. JIP1 also selectively interacts with the MLK family of MAP3K. MKK7 and MLK bind to regions on JIP1 that are distinct from the JNK binding site (Whitmarsh *et al.*, 1998). HPK1 also interacts with JIP1; however, whether this interaction is direct or mediated through MLK family members is uncertain (Whitmarsh *et al.*, 1998). These results strongly suggest that JIP1 serves as a scaffold protein in mammalian cells like Ste5p in yeast cells.

Mammalian MAP kinase pathways are much more complicated than those of yeast. It is unknown whether each kinase cascade is coordinated by a specific scaffold protein. Several pieces of evidence suggest that some mammalian kinases may serve as scaffold proteins, similar to Pbs2p in yeast. For example, MEKK1 is capable of interacting with JNK (Xu and Cobb, 1997), MKK4 (Su *et al.*, 1997), NIK (murine HGK) (Su *et al.*, 1997), and HPK1 (Hu *et al.*, 1996) through different regions of the MEKK1 protein. These properties enable MEKK1 to serve as a scaffold protein. However, whether MEKK1 can bind to all of these kinases simultaneously and the contribution of this binding to signaling specificity is unknown. Several pieces of evidence suggest that certain stimuli may use distinct kinase cascades to activate JNK through signal-specific scaffold proteins. For example, TAK1-induced JNK activation can be blocked by a dominant-negative mutant of MKK4 or MKK7, suggesting that both MKK4 and MKK7 have the potential to mediate TAK1-induced JNK activation (Zhou *et al.*, 1999). However, TGF-β-induced JNK activation, which is mediated by TAK1, is blocked by the dominant-negative mutant of MKK4, but not by a MKK7 mutant (Zhou *et al.*, 1999). These data suggest that TGF-β signaling specifically uses the TAK1-MKK4-JNK cascade, but not the TAK1-MKK7-JNK cascade, implying a signal-specific scaffold may be involved.

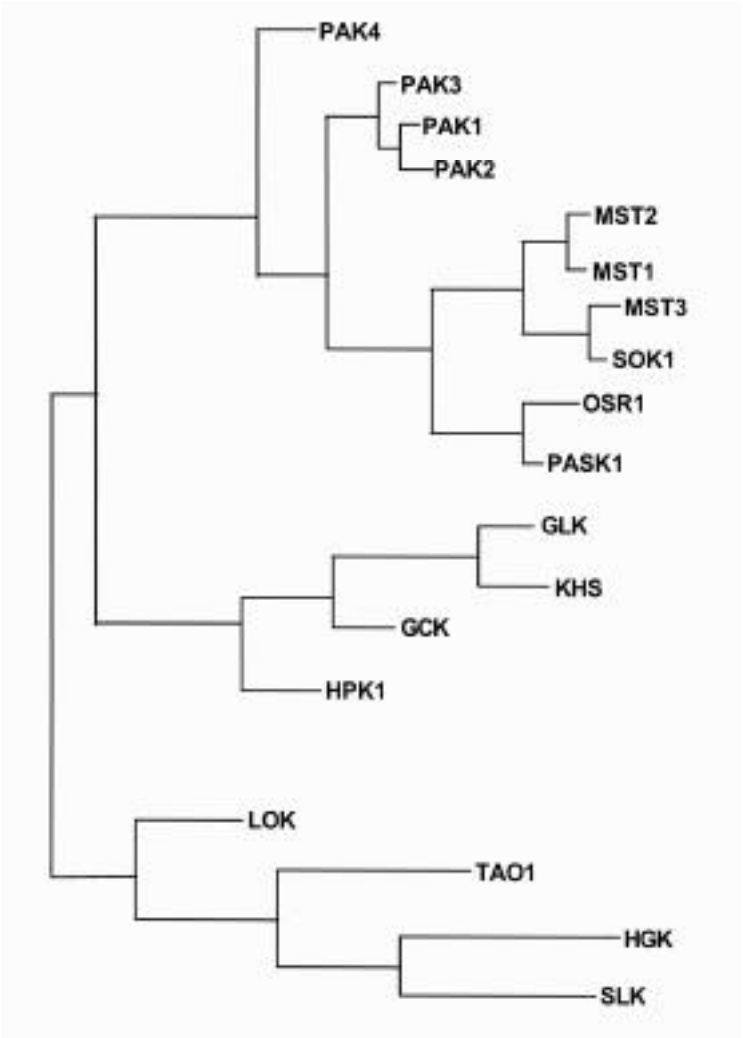

Figure 4. Relationship between members of mammalian STE20-related kinases. The phylogenetic analysis was performed using the multiple alignment server provided by the DNA DataBank of Japan (malign@nig.ac.jp). Human kinase sequences are used in these analyses, except PASK (rat), SLK (murine), and TAO1 (rat).

VII. Conclusion

The c-Jun N-terminal kinase pathway is activated by a variety of extracellular and intracellular stimuli. The biochemical mechanisms by which these stimuli converge upon and regulate the JNK pathway are intriguing. The discovery of multiple upstream JNK regulators, especially at the MAP3K and MAP4K levels, suggests that these kinases may connect the JNK signaling module to the upstream signals. However, the involvement and requirement of these kinases in JNK activation by specific stimuli remains unclear. To date, the studies on these kinases rely upon transient transfection assay, or on the examination of the activation of endogenous kinases by certain stimuli. The dominant-negative kinase mutants are useful to determine the possible involvement of these kinases in response to a specific stimulus; however, the establishment of genetically deficient animals or cell lines would be extremely critical to elucidate the biochemical and physiological importance of these JNK activators. In addition, revealing the further upstream regulators that link MAP3Ks and MAP4Ks to stimuli will be important.

JNK isoforms appear to have different substrate specificity. MKK4 and MKK7 also seem to have different substrate specificity, and are differentially regulated by upstream activators. The expanding molecules in the MAP3K and MAP4K levels further create complexity and diversity in the signaling specificity. The identification of scaffold-like proteins, such as JIP1, in mammalian cells indicates that the signaling molecules in the MAPK pathways may form complexes through interactions with scaffold proteins. Different complexes, with distinct components, may mediate different upstream signals and regulate distinct downstream effectors. Although the emergence of the scaffold model provides an explanation for the control of signaling specificity, many questions arise. Does the formation of these complexes occur before or after the stimulation? Is the specificity of these scaffold proteins stringent, or is it relatively flexible? Are these signaling complexes stable, or can the components in different complexes be exchanged rather freely? The answers to these questions will greatly enhance the understanding of cellular signaling.

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