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Structural organization of MAP-kinase signaling modules by scaffold proteins in yeast and mammals

Alan J. Whitmarsh and Roger J. Davis

MAP-kinase signaling pathways are activated by multiple extracellular stimuli. The specificity of activation and function of MAP-kinase signaling modules is determined, in part, by scaffold proteins that create multi-enzyme complexes. In *Saccharomyces cerevisiae*, two MAP-kinase-scaffold proteins have been identified. Recent studies of mammalian cells have also led to the identification of putative scaffold proteins. These scaffold proteins appear to facilitate MAP-kinase activation, in response to specific physiological stimuli, and to insulate the bound MAP-kinase module against activation by irrelevant stimuli. Scaffold proteins are therefore critical components of MAP-kinase modules and ensure signaling specificity.

MITOGEN-ACTIVATED PROTEIN (MAP) kinases phosphorylate Ser-Pro and Thr-Pro motifs in substrate proteins and are regulated by numerous extracellular stimuli, including growth factors, mitogens, cytokines and environmental stress¹. MAP-kinase signaling pathways have been implicated in the regulation of the physiological responses of many organisms (e.g. plants, fungi, slime moulds, insects and mammals)^{1–3}. In the

budding yeast *Saccharomyces cerevisiae*, MAP-kinase signaling pathways control diverse cellular processes, such as sporulation, cell-wall integrity, invasive growth, pseudohyphal growth, osmoregulation and mating². In *Drosophila melanogaster*, MAP-kinase signaling pathways are required for embryonic development and the immune response³, whereas in mammalian cells these signaling pathways have been implicated in cell growth, oncogenic transformation, cell differentiation and apoptosis^{1,3}.

MAP-kinases are activated by dual phosphorylation on threonine and tyrosine residues within the T-loop adjacent to the catalytic cleft by a signaling module that includes a MAP-kinase kinase (MKK) and a MAP-kinase-kinase kinase

(MKKK)¹. Distinct signaling modules activate different MAP-kinase groups. However, these groups can be activated by overlapping sets of extracellular stimuli, and some components of these modules can participate in more than one signaling pathway. Therefore, mechanisms that allow cells to achieve signaling specificity and to respond correctly to changes in the extracellular environment must exist. Indeed, in *S. cerevisiae*, genetic analysis has demonstrated that the different MAP-kinase modules are functionally distinct, irrespective of whether they use the same protein kinases⁴.

One important mechanism that regulates signal transduction pathways is the formation of complexes, either between different components of particular signaling pathways or between signaling molecules and anchor or scaffold proteins⁵. Anchor proteins localize their binding partners to specific subcellular compartments or to specific substrates, whereas scaffold proteins can bind several signaling molecules to create multi-enzyme complexes. These proteins include components of the I- κ B-kinase complex, the receptor for activated C kinase (RACK) group of proteins, and the A-kinase-anchoring protein (AKAP) family⁵. Scaffold proteins have also been identified for two *S. cerevisiae* MAP-kinase pathways: the pheromone mating-response pathway; and the osmoregulatory pathway.

The scaffold protein Ste5p coordinates the yeast MAP-kinase module that regulates mating

The Ste5p scaffold binds components of the pheromone mating-response MAP-kinase module (Fig. 1). Distinct regions of Ste5p interact with the MKKK Ste11p, the MKK Ste7p, and the MAP-kinases Fus3p and Kss1p (Ref. 6). These protein kinases co-sediment with Ste5p

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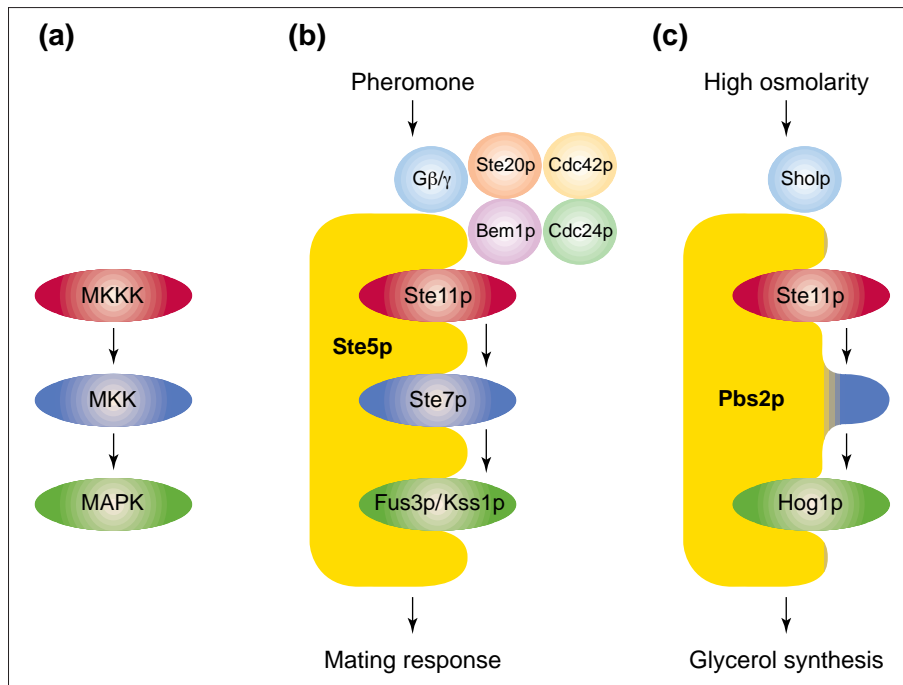


Figure 1

MAP-kinase scaffold complexes in *Saccharomyces cerevisiae*. **(a)** The generalized MAP-kinase module. **(b)** The phomone mating-response MAP-kinase pathway comprises the MAP-kinase-kinase kinase (MKKK) Ste11p, the MAP-kinase kinase (MKK) Ste7p, and the MAP kinase Fus3p (and possibly also Kss1p). This MAP-kinase cascade is coordinated by the scaffold protein Ste5p. The Ste5p scaffold also interacts (directly and indirectly) with additional components of this signaling pathway, including the G-protein subunits Gβ (Ste4p) and Gγ (Ste18p), Ste20p, Cdc42p, Cdc24p and Bem1p. **(c)** A second scaffold protein, the MKK Pbs2p, also exists in yeast. This protein kinase interacts with the transducer Sho1p, Ste11p and the MAP kinase Hog1p to create a MAP-kinase signaling module that regulates the biosynthesis of the osmotic stabilizer glycerol in response to exposure to osmotic stress. Sln1p is an independent osmosensor (not shown) that leads to the activation of two functionally redundant MKKKs (Ssk2p and Ssk22p) that activate Pbs2p independently of Ste11p.

in glycerol gradients, which suggests that they can bind Ste5p simultaneously, forming a high-molecular-weight complex⁶. This Ste5p signaling complex is present in the absence of signal. Upon phomone stimulation, Ste5p facilitates signal transduction from Ste11p to Fus3p and Kss1p. The activated MAP kinases initiate cellular processes necessary for mating, including transcriptional activation of mating-specific genes, cell-cycle arrest in G1 phase, polarized growth and cell fusion⁶. Fus3p and Kss1p are genetically redundant for mating. However, unlike Fus3p, which is present only in haploid cells, Kss1p is present in haploid and diploid cells, which suggests that it has other functions. Indeed, a recent report implicates Fus3p as the mating-pathway MAP kinase and suggests that Kss1p only functions in the mating pathway in the absence of Fus3p (Ref. 4). In wild-type haploid cells, Kss1p regulates invasive growth, whereas in diploids Kss1p regulates a similar filamentation process, pseudo-hyphal development. In Fus3p-deleted

strains, Kss1p operates in both the mating and filamentation signaling pathways. This causes incorrect biological responses to mating phomone⁴.

Recent studies indicate that other proteins also associate with the Ste5p complex. These include the heterotrimeric G-protein subunit Gβ (Ste4p) and Bem1p, a regulator of cell polarity. The binding of phomones to G-protein-coupled receptors causes the dissociation of the Gβγ subunits (Ste4p and Ste18p) from the inhibitory Gα subunit (Gpa1p). The Gβ subunit of the Gβγ dimer can bind to Ste5p^{7,8} and the protein kinase Ste20p (Ref. 9). Genetic studies have placed Ste20p upstream of Ste11p in the phomone signaling pathway, and Ste20p is thought to activate Ste11p (Ref. 6). Ste20p associates with the Ste5p signaling complex, but it is not clear whether it can bind directly to the scaffold protein. *In vitro*, Ste20p can phosphorylate Ste11p, but this phosphorylation does not alter Ste11p activity, which suggests that additional proteins and/or processes are involved⁶.

Gβ binds to the N-terminal LIM domain of Ste5p, but the mechanism of Gβ-induced signaling through Ste5p is unclear. Inouye *et al.*⁷ have proposed that the binding of Gβ to Ste5p is required for oligomerization of Ste5p – a prerequisite for signal transduction. However, Feng *et al.*⁸ have constructed a mutant Ste5p that is defective in Gβ binding but does oligomerize and facilitate signaling from Ste11p to Fus3p. The binding of Gβ to Ste5p appears to be essential for activation of Ste11p. The function of Gβ binding might therefore be to localize Ste11p to the plasma membrane where it can be activated^{8,10}.

Bem1p is an SH3-domain-containing protein that binds Cdc24p, an essential guanine-nucleotide-exchange factor for the Rho-family GTPase Cdc42p. Bem1p, Cdc24p and Cdc42p are proteins that mediate polarized growth during budding and the formation of mating projections (shmooing). They localize near cortical actin patches at growth sites and regulate the actin cytoskeleton. Bem1p binds both Ste5p and Ste20p, coordinates phomone-induced MAP-kinase activation with G1 arrest, and acts as a physical link between regulators of cell polarity and the Ste5p signaling complex^{11,12}. Interestingly, Bem1p also associates with the Fus3p substrate Far1p, which is required for both G1 arrest and partner selection during mating. This suggests that, in addition to linking the Ste5p–MAP-kinase complex to upstream activators, Bem1p also connects this MAP-kinase module to downstream MAP-kinase substrates¹².

Although genetic and biochemical evidence support a role for Ste5p as a scaffold protein, it remains unclear how the scaffold function of Ste5p is regulated. Oligomerization of Ste5p is required for Ste5p function, and dimerization domains are present within the N-terminus of the protein¹³. Ste5p is a phosphoprotein, and is phosphorylated by the MAP kinase that it binds (Fus3p) and by additional protein kinase activities⁶. The effect of phosphorylation on Ste5p-mediated signal transduction is unknown. Phosphorylation of Ste5p by Fus3p could destabilize the complex and thereby act as negative feedback. The location of Ste5p within the cell is likely to be critical for its function. It is not clear whether Ste5p is restricted to the cytoplasm or can translocate to the nucleus upon activation. Fus3p phosphorylates both cytoplasmic and nuclear substrates. Once activated, Fus3p might dissociate

from Ste5p and phosphorylate its substrates. Alternatively, Ste5p might localize Fus3p to specific substrates. Further studies are required if we are to define the detailed mechanisms by which the Ste5p signaling complex regulates the mating response.

Scaffold protein Pbs2p coordinates an osmoregulatory MAP-kinase module in yeast

Pbs2p is representative of a class of scaffold proteins that is distinct from Ste5p (Fig. 1c). Pbs2p coordinates an osmoregulatory MAP-kinase module in *S. cerevisiae* and, in contrast to Ste5p, is a protein kinase component of the MAP-kinase cascade for which it serves as a scaffold¹⁴. In response to high osmolarity, two independent osmosensors (Sln1p and Sho1p) relay signals to the MAP kinase Hog1p, which leads to increased synthesis of the osmotic stabilizer glycerol. The Sln1p osmosensor is homologous to the two-component signal transducers found in bacterial signaling pathways and utilizes a multistep phosphorelay mechanism to regulate the functionally redundant MKKKs Ssk2p and Ssk22p, which phosphorylate and activate the MKK Pbs2p, which in turn phosphorylates and activates Hog1p (Ref. 15). The second osmosensor, Sho1p, activates a cascade consisting of Ste11p, Pbs2p and Hog1p (Ref. 14). Pbs2p forms a multiprotein complex with Sho1p, Ste11p and Hog1p, but not Ssk2p or Ssk22p. The SH3 domain of Sho1p binds to an N-terminal proline-rich domain in Pbs2p; this interaction is required for the activation of this signaling pathway in response to high osmolarity¹⁴. Whether the binding of Sho1p, Ste11p and Hog1p to Pbs2p occurs simultaneously remains to be determined.

Scaffold proteins confer specificity on MAP-kinase signaling modules

The colocalization of successive members of a MAP-kinase cascade on the Ste5p and Pbs2p scaffold proteins has two important advantages: (1) it favors the rapid passage of the signal through the cascade; and (2) it prevents unwanted crosstalk with components of other MAP-kinase pathways. The latter is clearly demonstrated by the finding that three yeast MAP-kinase modules share common protein kinases but regulate distinct biological processes (Fig. 2). Ste20p, Ste11p and Ste7p are shared by the pheromone-response pathway and the invasive-growth pathway⁴. Ste11p is also shared with the Sho1p-mediated

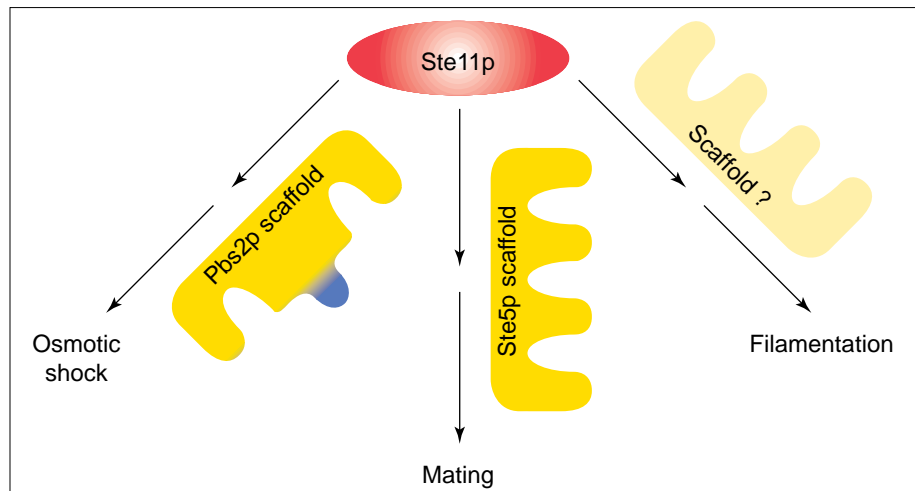


Figure 2

Scaffold proteins specify and insulate the signaling functions of MAP-kinase modules. The yeast MAP-kinase kinase (MKKK) Ste11p participates in three distinct MAP-kinase signaling modules: the Pbs2p scaffold coordinates components of an osmoregulatory MAP-kinase signaling module; the Ste5p scaffold coordinates components of the pheromone-response MAP-kinase signaling module; and Ste11p also participates in a MAP-kinase signaling module that regulates filamentation. A scaffold protein that functions within the filamentation MAP-kinase signaling module has not been identified. This MAP-kinase module might therefore function independently of a scaffold protein.

osmoregulatory pathway¹⁴. Thus, Ste11p functions within three separate MAP-kinase modules: (1) in response to pheromone, the Ste5p scaffold restricts Ste11p to phosphorylating Ste7p (not Pbs2p), which leads to Fus3p MAP-kinase activation; (2) in response to high osmolarity, Pbs2p forms a complex with and is phosphorylated by Ste11p, which leads to Hog1p MAP-kinase activation; and (3) in the invasive-growth response, Ste11p activates Ste7p (perhaps by a scaffold-independent mechanism), which leads to Kss1p MAP-kinase activation (Fig. 2).

Independent MAP-kinase signaling pathways can be coordinated by scaffold proteins

Although MAP-kinase signaling pathways can be regulated independently, correct biological responses probably require the coordinated actions of more than one MAP-kinase signaling pathway. The response of yeast to pheromone is an example. Whereas the Fus3p signaling pathway is important for many aspects of the mating response, the polarized growth of mating projections (shmooing) involves new cell-wall biosynthesis, which requires activation of the cell-integrity MAP kinase Mpk1p. Mating therefore requires the coordination of two different MAP-kinase pathways. Thus, mating-pathway activation leads to new gene expression and subsequent activation of the Mpk1p pathway¹⁶. The

mechanism that coordinates these MAP-kinase pathways is unclear. However, genetic analysis suggests that Spa2p plays an important role in this process^{16,17}. Spa2p localizes to sites of polarized growth during budding, mating and pseudohyphal growth, and forms complexes with the coiled-coil protein Pea2p and the actin-binding protein Bud6p (Ref. 17). Spa2p also binds to activators of both the Fus3p pathway (Ste7p) and the Mpk1p pathway (Mkk1p and Mkk2p)¹⁷. In addition, weak interactions with the MKKK Ste11p have been detected for both Spa2p and Bud6p. The functional significance of these interactions has not been established. However, these observations suggest that Spa2p is a scaffold protein that assists the coordination of the Fus3p and Mpk1p pathways. Spa2p might function by localizing MAP-kinase activation to sites of polarized growth. Furthermore, as the cell-integrity-pathway MKKK Bck1p does not appear to be required for Mpk1p activation in response to pheromone (at least in some strains), one function of Spa2p might be to facilitate the activation of Mpk1p by the mating-pathway MKKK Ste11p. Further studies are required if we are to establish the mechanism of action of Spa2p.

Whether Spa2p is representative of a larger class of regulatory molecules that control MAP-kinase function has not been established. Interestingly, however,

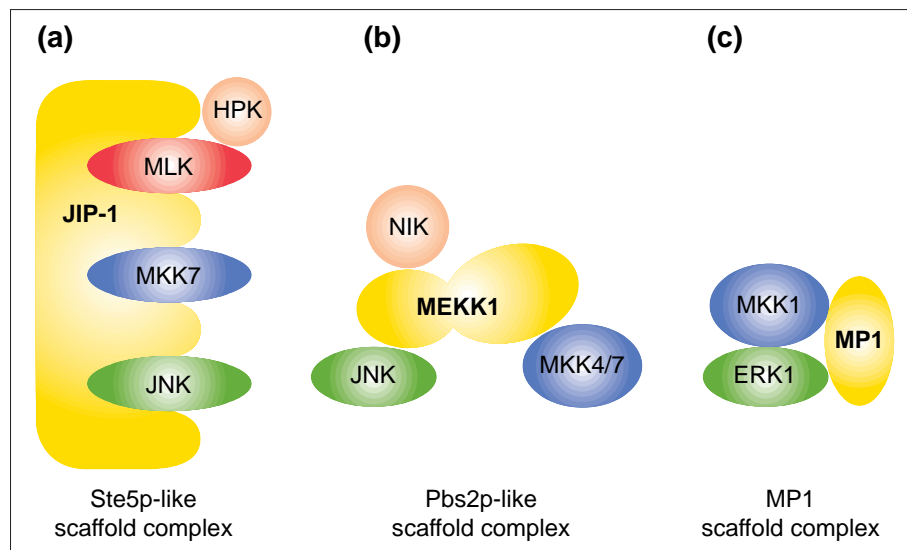


Figure 3

Mammalian MAP-kinase-scaffold complexes. Three classes of mammalian MAP-kinase-scaffold protein have been identified. **(a)** The JNK-interacting protein (JIP-1) appears to coordinate components of the JNK MAP-kinase module. The JIP-1 scaffold complex includes the Ste20p-like protein kinase HPK-1, the MLK group of MAP-kinase-kinases (MKKK), the MAP-kinase kinase (MKK) isoform MKK7, and the MAP-kinase JNK. The mammalian complex coordinated by JIP-1 and the yeast MAP-kinase complex coordinated by Ste5p share similar features, including interaction with multiple components of the MAP-kinase module, facilitation of MAP-kinase activation in response to specific signals, and insulation of the bound MAP-kinase module against activation by irrelevant stimuli. **(b)** The second class of mammalian MAP-kinase scaffold complex is represented by the MKKK isoform MEKK1, which interacts with the Ste20p-like protein kinase NIK, the MKK protein kinases MKK4, and the MAP kinase JNK. Whether this complex has any physiological significance remains to be established. The putative mammalian scaffold complex coordinated by MEKK1 shares similar properties with the Pbs2p-coordinated scaffold complex that exists in yeast. **(c)** The protein MP1 represents a third class of mammalian scaffold protein. MP1 binds to the MAP kinase ERK1 and to the MKK isoform MKK1. MP1 appears to function by facilitating the activation of ERK1 by MKK1. A protein that has properties that are similar to those of MP1 has not been identified in yeast.

in yeast, the Spa2p-related protein Sph1p also appears to interact with Ste11p, Ste7p, Mkk1p and Mkk2p (Ref. 18). In addition, proteins related to Spa2p have been detected in nematodes and humans¹⁷. Further studies are required to determine whether the physiological functions of these proteins are also related.

MAP-kinase scaffold proteins in mammals

It has been unclear whether Ste5p- or Pbs2p-like scaffold proteins exist in mammalian cells. However, recent studies indicate that such proteins do function in mammalian cells (Fig. 3). A candidate scaffold protein that might facilitate signaling by a stress-activated MAP-kinase pathway has been identified. JNK-interacting protein 1 (JIP-1) was isolated in a two-hybrid screen for proteins that bind to the MAP kinase JNK (Ref. 19). JIP-1 is a cytoplasmic protein that is ubiquitously expressed, although predominantly in brain, testis, kidney and lung. JIP-1 selectively binds to JNK (but not to the ERK or p38 MAP

kinases) with an affinity 100-fold greater than the affinity of JNK for its substrate, JUN. JIP-1 was characterized initially as an inhibitor of JNK signaling, by virtue of its ability to prevent nuclear translocation of JNK. Overexpression of JIP-1 in cells thereby prevented JNK-mediated gene expression, suppressed JNK-mediated transformation by the BCR-ABL oncogene and blocked JNK-mediated apoptosis in response to nerve-growth-factor withdrawal from PC12 cells¹⁹.

Further characterization of JIP-1 (Ref. 20) has revealed that JIP-1 selectively interacts with multiple components of the JNK signaling pathway (Fig. 3a). JIP-1 binds the MKK isoform MKK7 (a specific activator of JNK) but not MKK4, which activates JNK and p38 MAP kinases. In addition, JIP-1 selectively interacts with the mixed-lineage protein kinase (MLK) family of MKKKs. MKK7 and MLK bind directly to regions of JIP-1 that are distinct from the JNK-binding site. Whether the MLK, MKK7 and JNK protein kinases interact simultaneously with JIP-1 remains

to be established. The Ste20p-like protein kinase HPK-1 also associates with JIP-1 in co-immunoprecipitation assays, but it is not clear whether HPK-1 binds directly to JIP-1 or whether this binding is mediated by MLK-family members. In transient transfection assays, JIP-1 increases JNK activation by the MLK–MKK7 signaling pathway. Specific extracellular signals that trigger JIP-1-mediated JNK activation, and the physiological targets of this MAP-kinase module, remain to be determined. Gel-filtration analysis demonstrates that MKK7 is present in high-molecular-weight complexes in mammalian cell extracts²¹, but we do not know whether all or only some of these complexes include JIP-1. Studies of the effect of JIP-1 gene disruption might be required to identify the biological significance of the JIP-1 scaffold complex.

Many questions concerning the role of JIP-1 remain to be resolved. For example, the mechanism of regulation and the precise cellular location of JIP-1 are not understood. Like Ste5p, JIP-1 is a phosphoprotein that is phosphorylated by the MAP kinase that it binds. The significance of this phosphorylation for the assembly of the complex needs to be determined. Whether JIP-1, like Ste5p, requires oligomerization to function efficiently is not known. In transfected cells, JIP-1 is exclusively located in the cytoplasm; however, further studies are required to determine where endogenous JIP-1 is located and whether JIP-1 translocates to a different cytoplasmic locale or to the nucleus in response to appropriate stimulation. Further questions relate to the observation that JIP-1 is expressed in cells as two alternatively spliced isoforms that differ by the insertion of 47 amino acid residues in the C-terminal region. The difference in the functions of these isoforms is unclear because both form complexes with HPK1, MLK3, MKK7 and JNK (Ref. 20). The fact that JIP-1 is a putative transcription factor that binds to a regulatory element in the *GLUT2* gene promoter is also interesting²². Further studies are required to determine whether JIP-1 is a protein that has multiple physiological functions or whether the role of JIP-1 as a MAP-kinase scaffold contributes to *GLUT2* gene expression.

Despite a number of unanswered questions, JIP-1 appears to fulfill some of the functional criteria established for the Ste5p scaffold protein. First, it selectively interacts with multiple

components of a signaling module. Second, it facilitates signaling by the bound protein kinases. Third, the selective inhibition of JNK signaling by JIP-1 is consistent with the predicted insulating function of a scaffold protein. Therefore, although JIP-1 bears no sequence similarity to Ste5p, it appears to have an analogous function.

A Pbs2p-like scaffold protein might also exist in mammalian cells (Fig. 3b). One candidate is MEKK1, a protein kinase that regulates MAP-kinase signaling pathways (ERK and JNK) and MAP-kinase-independent signaling events²³. Recent studies have demonstrated that the N-terminus of MEKK1 associates with NIK (a Ste20p-like protein kinase) and JNK. NIK also binds to the adaptor protein NCK, a target for several growth factor receptors, and might therefore link these receptors to the JNK pathway²⁴. JNK binds directly to the N-terminus of MEKK1; this interaction can potentiate JNK activation by a mechanism that does not require the C-terminal kinase domain of MEKK1 (Ref. 25). However, the C-terminal domain of MEKK1 does bind MKK4 (Ref. 25) and RAS (Ref. 23), although the functional significance of these binding events is not clear. Further studies are required to determine whether MEKK1 is in fact a scaffold protein similar to Pbs2p and whether other mammalian MEKK-family members also perform scaffold functions for their respective MAP-kinase modules.

A third class of MAP-kinase-scaffold complex has recently been identified in mammalian cells (Fig. 3c). The protein MP1 is a representative member of this group²⁶. The MKK isoform MKK1 and ERK1 bind to MP1. Interestingly, MP1 does not interact with MKK2 or ERK2. The MP1 scaffold complex appears to facilitate the activation of ERK1 by MKK1. Whether this scaffold complex includes additional components of the ERK MAP-kinase pathway is not clear.

Other potential mammalian scaffold proteins that might regulate MAP-kinase signaling pathways also exist. One example is the kinase suppressor of RAS (KSR). Genetic studies in *Drosophila* and *Caenorhabditis elegans* led to the isolation of KSR as a protein required for RAS signaling²⁷. KSR bears some sequence similarity to the MKKK RAF-1: it possesses a cysteine-rich domain in the N-terminal region and a potential kinase domain in the C-terminal region. The precise role of KSR in RAS signaling is not clear. Zhang *et al.*²⁸ have reported

that KSR is a ceramide-activated protein kinase that, in the presence of ceramide or treatment with TNF α , associates with, phosphorylates and activates RAF-1. More-recent reports do not substantiate this finding^{29,30}. Furthermore, Yu *et al.*³⁰ demonstrate that KSR binds both the MKK isoform MEK1 and the MAP kinase ERK2, and propose that KSR acts as scaffold protein that links MEK1 to its substrate ERK2.

Other proteins that associate with components of MAP-kinase signaling pathways in mammals include the adaptor protein GRB10 and cytoskeletal proteins, such as actin-binding protein 280 (ABP-280) and KIF3. The SH2 domain of GRB10 binds to receptor tyrosine kinases and, in a phosphotyrosine-independent manner, GRB10 binds to RAF-1 and MEK1 (Ref. 31). The binding of GRB10 to RAF-1 appears to be constitutive, whereas binding to MEK1 requires cell stimulation and activation of ERK. Whether GRB10 has a positive or negative regulatory role in signaling through the ERK MAP-kinase pathway remains to be established. ABP-280 binds to MKK4 and is required for maximal TNF α -induced activation of JNK in melanoma cells³², whereas the MLK-family member MLK2 colocalizes with activated JNK along microtubules by associating with the kinesin motor protein KIF3 (Ref. 33). The functional significance of this interaction is not clear. Further studies are required to examine the role of these putative scaffold proteins in the regulation of mammalian MAP-kinase signaling modules *in vivo*.

Conclusions

Increasing evidence supports a role for scaffold proteins in the regulation of MAP-kinase signaling pathways in yeast and mammals. In *S. cerevisiae*, scaffold-dependent and scaffold-independent mechanisms modulate the activity of particular MAP-kinase signaling pathways (Fig. 2). In mammals, information concerning MAP-kinase-scaffold proteins is more limited. However, potential mammalian MAP-kinase-scaffold complexes have been described, and additional mammalian scaffold complexes should soon be defined. Understanding the physiological roles of such scaffold complexes in restricting the subcellular location of MAP-kinase signaling pathways, targeting MAP kinases to specific substrates, or linking these signaling pathways to specific cellular events will be of particular importance. Rapid

progress towards gaining answers to these questions should be made during the next few years.

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