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The Ste20 group kinases as regulators of MAP kinase cascades

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Ste20p (sterile 20 protein) is a putative yeast mitogen-activated protein kinase kinase kinase kinase (MAP4K) involved in the mating pathway. Its homologs in mammals, *Drosophila*, *Caenorhabditis elegans* and other organisms make up a large emerging group of protein kinases including 28 members in human. The Ste20 group kinases are further divided into the p21-activated kinase (PAK) and germinal center kinase (GCK) families. They are characterized by the presence of a conserved kinase domain and a noncatalytic region of great structural diversity that enables the kinases to interact with various signaling molecules and regulatory proteins of the cytoskeleton. This review describes the phylogenetic relationships of the Ste20 group kinases based on discussions with many researchers in this field. With the newly established phylogenetic relationships, crucial arguments can be advanced regarding the functions of these kinases as upstream activators of the MAPK pathways and possible activity as MAP4Ks. Their involvement in apoptosis, morphogenesis and cytoskeletal rearrangements is also discussed.

The group of kinases that is related to the budding yeast Ste20p (sterile 20 protein) kinase has recently received considerable attention. There are about 30 Ste20-related kinases in mammals in addition to homologs in *Drosophila*, *Caenorhabditis elegans* and other organisms. The group includes germinal

center kinases (GCKs) and p21-activated kinases (PAKs), which have various intracellular regulatory effects including the regulation of apoptosis and rearrangement of the cytoskeleton leading to cell-shape change and cell motility^{1–3}.

Recent findings that most Ste20 group kinases activate the mitogen-activated protein kinase (MAPK) cascades have gained further interest^{1–3}. The MAPK cascades are crucial in a wide range of cellular events, transmitting signals from extracellular stimuli such as growth factors, cytokines and environmental stresses to activate transcription factors, resulting in regulation of gene expression^{4,5}. The signaling is mediated by linear sequential phosphorylation of a triple-kinase module consisting of MAP kinase kinase kinase (MAP3K), MAP kinase kinase (MAP2K) and MAPK^{4,5}. The triple-kinase module and its activation mechanism are highly conserved in the eukaryotic evolution from yeast to mammals^{4,5}. In this respect, especially noteworthy is the discovery that the yeast Ste20p activates a yeast MAP3K Ste11p by direct phosphorylation to mediate the

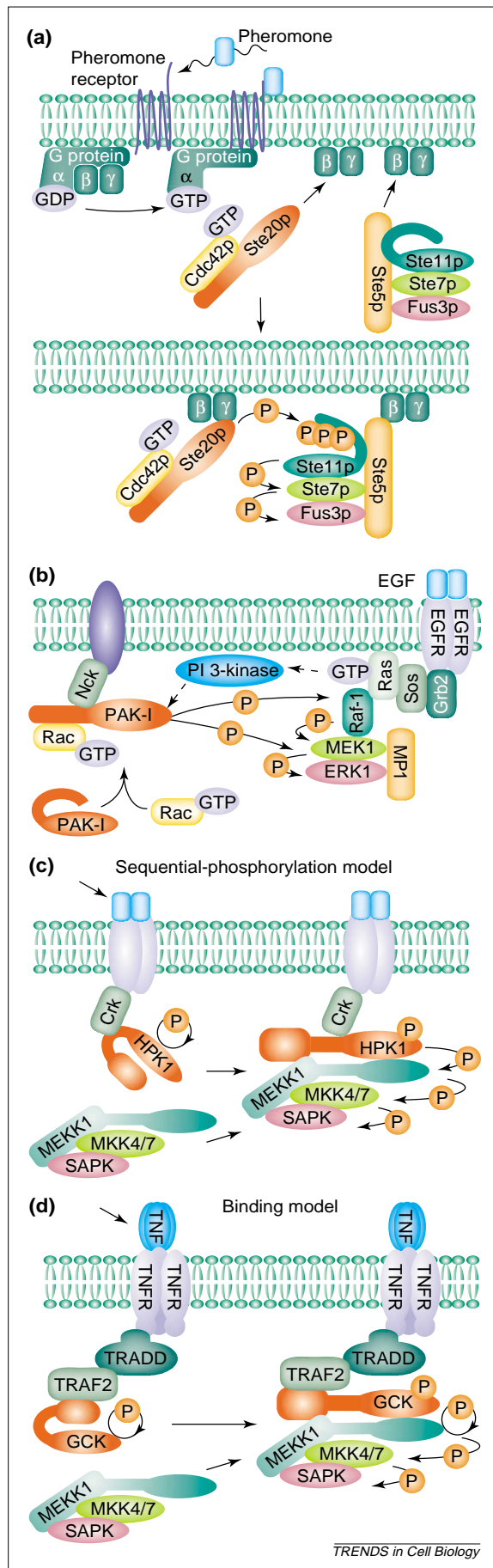


Fig. 1. The role of Ste20 group kinases as upstream activator of mitogen-activated protein kinase (MAPK) signaling pathways. (a) MAPK kinase kinase (MAP4K) activity of yeast Ste20p. Ste20p is involved in the mating pathway in the haploid budding yeast *Saccharomyces cerevisiae* as a MAP4K⁸. Binding of a peptide pheromone to a pheromone receptor activates a heterotrimeric G protein^{10,11}. First, GDP-GTP exchange releases Gβ (Ste4p) and Gγ (Ste16p) from the Gα (Gpa1p) subunit^{10,11}. Second, the MAPK module scaffolded by Ste5p is recruited to Gβ in a pheromone-dependent manner^{10,11}. Meanwhile Ste20p, which has been activated by Cdc42p binding, is also recruited to Gβ⁹. It is not clear whether the two signaling components are recruited to the same Gβ subunit (to form a Gβ-Ste20p-MAPK complex) or different Gβ subunits (to form Gβ-Ste20p and Gβ-MAPK complexes). Ste20p in turn phosphorylates the yeast MAP3K Ste11p, disrupting its intramolecular association between the kinase domain and the noncatalytic region^{6,7}. Ste11p is associated with Ste7p (MAP2K) and Fus3p or Kss1p (MAPKs) with the help of the scaffold protein Ste5p⁶⁴. The sequential phosphorylation of these kinases within the complex leads to the activation of MAPK, which migrates into the nucleus to activate transcription factors that regulate the transcription of the genes involved in the mating reaction⁸. (b) MAP4K activity of members of the p21-activated kinase (PAK-I) subfamily in the extracellular-signal-regulated kinase (ERK) MAPK pathway. PAK-I subfamily members probably mediate the signal from Ras to the ERK pathway in an indirect way^{1,2,17}. A ligand-activated tyrosine kinase recruits the GDP-GTP exchange factor Sos through the adaptor protein Grb2¹. Sos induces the exchange of Ras-bound GDP with GTP on the newly recruited Ras¹. The activated Ras recruits an MAP3K Raf-1 and activates it through unknown mechanisms¹. Raf-1 activates the MAP2K MEK1, which in turn activates MAPK ERK1¹⁴. ERK1 and MEK1 are both associated with the scaffold protein MP1⁶⁵. PAK-I subfamily members are probably involved in the indirect Raf-1 activation by Ras involving phosphoinositide 3-kinase (PI 3-kinase), in which PAK-I subfamily members are activated by GTP-bound Rac1 or Cdc42¹⁷. PAK-I subfamily members might activate the ERK MAPK pathway at two different stages: Raf-1 phosphorylation or MEK1 phosphorylation^{18,19}. (c) Sequential phosphorylation model. Hematopoietic progenitor kinase 1 (HPK1) activates its effector MAP3Ks by direct phosphorylation as an MAP4K^{14,15}. The upstream signaling component in the membrane has not been identified, but it is likely to be one of the receptor tyrosine kinases. Directly upstream, HPK1 interacts with several Src-homology 3 (SH3)-containing adaptor proteins (Crk is shown)⁶⁶. The activation mechanism of HPK1 has not been elucidated but based on the observations that HPK1 undergoes autophosphorylation and that the kinase domain and CNH domains of HPK1 are similar to those of germinal center kinase (GCK)^{14,15}, we anticipate that HPK1 might undergo conformational change upon autophosphorylation to make it accessible to its effector MAP3Ks (MEK1 is shown). The activated HPK1 phosphorylates MEKK1 to trigger the sequential phosphorylation of the stress-activated protein kinase (SAPK) signaling module consisting of MEKK1, MKK4/7 (MAP2K) and SAPK (MAPK) to result in the activation of SAPK^{14,15}. (d) Binding model. GCK-I subfamily members play important roles in mediating the signal from cytokine receptors such as tumor necrosis factor receptor α (TNFRα) to the SAPK pathway³. GCK plays an essential role in this cascade and the binding model better represents its function²¹. The binding of TNF to TNFRα homotrimer triggers recruitment of the TNFR-associated death domain protein (TRADD), which in turn recruits TNFR-associated factor 2 (TRAF2)³. GCK is recruited to TRAF2 and undergoes conformational change upon autophosphorylation to make it accessible to its effector MAP3Ks (MEKK1 is shown)²¹. The bound MEKK1 is activated upon autophosphorylation^{28,29}, and phosphorylates MAP2K (e.g. MKK4 or 7), which in turn phosphorylates SAPK^{4,5}.

signal from the mating pheromone receptor to the MAPK pathway^{6,7}. This demonstrates that Ste20p acts as an MAP kinase kinase kinase (MAP4K) (Fig. 1a), raising the interesting possibility that the mammalian homologs of Ste20p also function as MAP4Ks. The MAPK pathways have been one of the major focuses in the study of intracellular signaling, but although the activation cascade downstream of MAP3K that leads to the

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Box 1. Nomenclature of the kinases

According to the nomenclature system proposed by Hanks and Hunter^a for protein kinases where the term 'superfamily' is reserved for describing the entire group of kinases with the classical 'eukaryotic protein kinase' catalytic domain, 'groups', 'families', and 'subfamilies' are used to designate progressive degrees of relatedness. Following this system, the most suitable name for the kinases evolutionarily related to Ste20 would be the mammalian 'Ste20 group kinases'. The Ste20 group would then be divided into p21-activated kinase (PAK) and germinal center kinase (GCK) families, and further into subfamilies.

GCKs, which are often regarded as Sps1 family kinases, are unlikely to be orthologous to the yeast Sps1p^b (Fig. 2 in main text), and thus the use of this term should be avoided. The yeast Ste20p and PAKs share the same evolutionary origin because they are highly homologous, even in the noncatalytic domain^{c-e}. It is often irrelevant to integrate yeast and mammalian proteins in the same phylogenetic analysis without careful evolutionary considerations. It should be remembered that the budding yeast is not a primitive form of animal but belongs to a different kingdom, fungi, and has evolved in its own direction.

The mixed-lineage kinase (MLK) family including MAP3Ks such as MLK3 and TAK1^f, which is distantly related to Ste20p (see Fig. 2 where MLK3 is included as an outgroup), is sometimes included in the Ste20 group. However, it should be excluded because of its distinct characteristics as an MAP3K and its slightly different signature sequence in subdomain VIII, xgtxaWMAPEv.

GCK-VIII subfamily members might well give rise to a distinct family such as a 'TAO family', given that they are phylogenetically more distant from other GCKs and that most of them act as genuine MAP3Ks. Time will tell whether they would be better integrated into the GCK family or form a TAO family (Fig. 2 in main text).

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regulation of transcription has been largely elucidated, the upstream signaling modules have remained obscure^{4,5}. The emergence of such a large group of kinases that can act as upstream regulators of the MAPK cascades and connect them to more diverse upstream signaling components, including membrane-associated receptors, has added a new dimension to the study of MAPK signaling pathways.

This review has three main aims. First, we establish the phylogenetic relationships among the Ste20 group kinases. Our extensive search of the EST databases and the draft human genome sequences on the public databases suggests that nearly all members of the mammalian Ste20 group kinases have been found. Nevertheless, the reorganization of the phylogenetic classification has not caught up with recent cloning efforts. Reorganizing the Ste20 group kinases based on phylogenetic relationships might suggest important new research directions. Our analysis indicates that the current classification of the Ste20 group kinases should be reorganized¹⁻³ and has revealed several distinct subfamilies in the group. The grouping system described in this review is the result of a series of e-mail conferences among the GCK-family kinase researchers, who agreed that a rough phylogenetic-based grouping system is better than a rigid nomenclature system (e.g. GCK = MAP4K2).

Second, we examine the possibility that Ste20 group kinases function as MAP4Ks in various facets of intracellular signaling. This examination shows that some Ste20 group kinases indeed activate MAPK cascades either by phosphorylating or by simply binding to MAP3Ks, but we warn against the simplistic view of regarding Ste20s as MAP4Ks in a linear sequential quadruple-kinase cascade.

Third, we review recent progress in understanding Ste20 group kinases based on phylogenetically established subfamilies. We concentrate on the Ste20 group kinases that function as upstream activators of MAPK pathways and those with roles in apoptosis, morphogenesis and the cytoskeletal regulation of cell shape and motility. We hope to show the current status and future possibilities in Ste20 group kinase research.

Overview of the mammalian Ste20 group kinases

Ste20p as a MAP4K in yeast

Ste20p was originally found as a key kinase in the mating pathway in the haploid budding yeast *Saccharomyces cerevisiae*⁶. This pathway is initiated by the binding of a peptide pheromone to a pheromone receptor, which activates a heterotrimeric G protein and leads to the activation of the yeast MAPK pathway consisting of Ste11p (MAP3K), Ste7p (MAP2K) and Fus3p and Kss1p (MAPKs) (Fig. 1a)⁸. The direct phosphorylation of

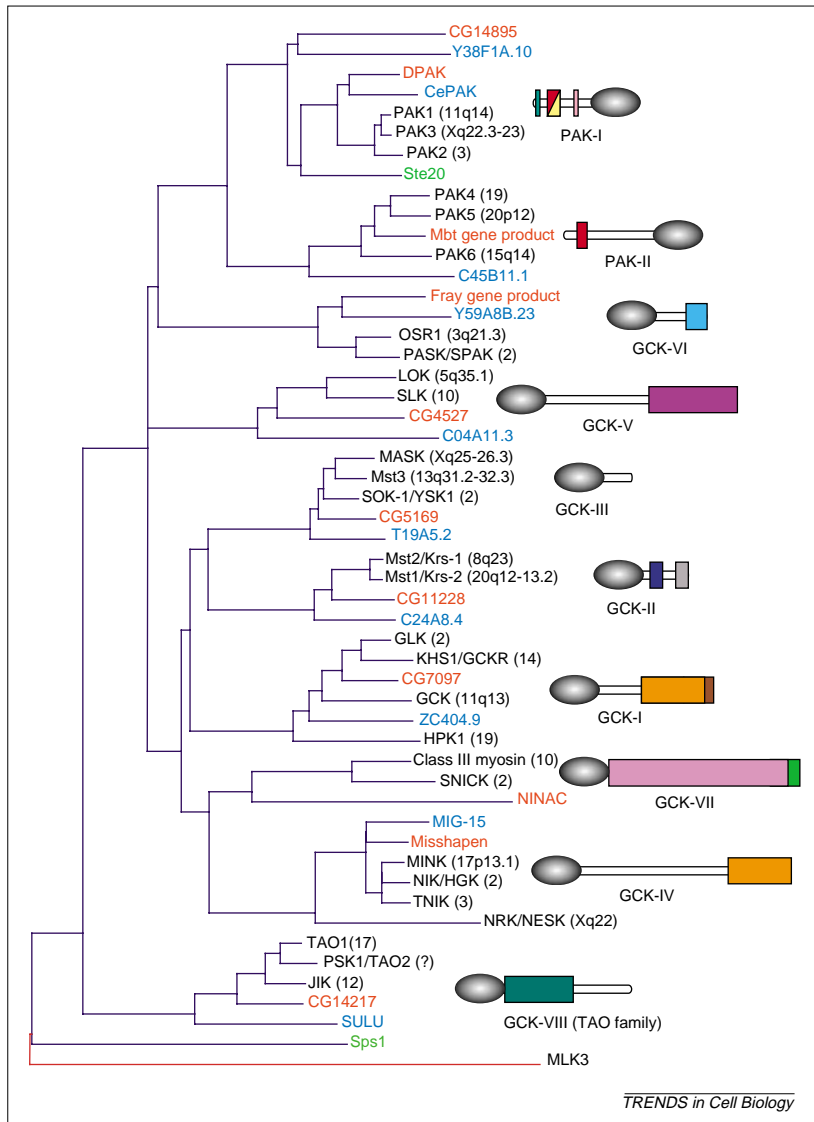


Fig. 2. Phylogenetic relations among mammalian Ste20 group kinases and their schematic structures in each subfamily. The amino acid sequence of the kinase domain of each Ste20 group kinase was obtained from human (black), *Drosophila* (red) and *Caenorhabditis elegans* (blue) databases. The chromosomal location of each human kinase is indicated in parentheses except for PAK1/TAO2 which is yet to be mapped. The budding yeast Ste20p and Sps1p are incorporated with the phylogenetic analysis to provide references (green)^{6,63}. We have not integrated other budding yeast Ste20 group proteins such as Cla4p, Pakp and Nrk1p² in order to limit our argument to Ste20 group kinases in animals. The sequences were aligned using a SAM program, and a phylogenetic tree was constructed with the neighbor-joining method using 10 000 bootstraps. The kinase domain sequence of MLK3 was used as an outgroup to generate a root for the phylogenetic tree. The schematic structure of each subfamily is shown: the black ovals represent kinase domains, the rectangles with different colors indicate conserved domains, and the white bars indicate the variable regions. Owing to space limitation, full names, aliases, orthologs in other animals, and their accession numbers on the DDBJ/EMBL/GenBank database cannot be shown here. They are available on our web site at <http://homepage2.nifty.com/genehunt>. The p21-activated kinase (PAK) family is marked by two common structural features: a kinase domain at the C-terminus and a Cdc42/Rac-1-binding (CRIB, red) domain in the middle of the N-terminal noncatalytic region^{1,2}.

Ste11p by Ste20p has recently been demonstrated to be a crucial step in the signaling pathway, showing that Ste20p can act as an MAP4K^{6,7}. Ste20p phosphorylates three amino acid residues of Ste11p (Fig. 1a)⁷. This disrupts an intramolecular interaction between the Ste11p N-terminal regulatory domain and its C-terminal catalytic domain, thus activating Ste11p⁷.

Activation of the Rho subclass of small G proteins, Cdc42 and Rac-1, induces modulation of the membrane-linked actin cytoskeleton, leading to changes in cellular morphology and motility¹. PAK-I subfamily members, consisting of PAK1, 2 and 3 (Box 2), have two characteristic motifs (Fig. 1). The first is the autoinhibitory domain (yellow) located downstream of the CRIB and slightly overlapping it¹. PAK-I subfamily members are inactivated by intramolecular interaction of the autoinhibitory domain with the kinase domain¹. The binding of Cdc42 or Rac to the CRIB domain inhibits this interaction, resulting in the activation of the kinase¹. The second is the Cool/Pix-binding motif (pink), which is crucial for the recruitment of PAK-I subfamily members to focal adhesion complexes upon Cdc42/Rac-1 stimulation¹. At the N-terminus, there is a proline-rich motif that serves as the binding site for the Src-homology 3 (SH3)-domain-containing adaptor protein Nck (light green)^{1,2}. The C-terminus region is implicated as a binding site for Gβ⁹. PAK-I subfamily members are highly homologous to Ste20p, sharing both a CRIB and autoinhibitory domains^{1,2}. They are so conserved that PAK3 can even complement *STE20* gene defects in the budding yeast⁶⁷. PAK-I subfamily members have a CRIB domain without a recognizable autoinhibitory domain, and lack a Cool/Pix binding motif¹³. The PAK-II CRIB sequences are slightly different from those of PAK-I, and bind Cdc42 with higher affinity than Rac¹³. Germinal center kinase (GCK)-family kinases have a conserved N-terminal kinase domain (in contrast to PAK-family kinases, which have a C-terminal kinase domain), but their noncatalytic regions exhibit a wide variety of structures. The GCK-I subfamily kinases have a highly variable intermediate region with several proline-rich motifs (potential SH3-binding sites, not shown specifically), a citron homology domain (CNH, orange), and a conserved C-terminal extension (brown)³. GCK-I family members interact with various molecules at their noncatalytic regions, including MAP3Ks (MEKK1, MLK3), the Rab8 GTPase, SH3-containing adaptor proteins (Grb2, Crk, CrkL, HIP-55, Gads), and tumor necrosis factor (TNF) receptor-associated factors (TRAF2, TRAF6)^{3,66,68,69}. GCK-IV subfamily members are structurally similar to those of GCK-I, and have a CNH that might mediate binding to MEKK1^{22-24,26,52,70,71}. However, homology between CNH of GCK-I and GCK-IV is rather weak (about 20%), and thus the same function might not be assigned (e.g. MEKK1 interacts with GCK-I and GCK-IV subfamily kinases at different sites³). The intermediate regions of GCK-IV are longer than those of GCK-I, and GCK-IV subfamily kinases lack the C-terminal extension^{22-24,26,52,70,71}. GCK-II subfamily kinases contain an autoinhibitory domain (dark blue) and a dimerization domain (gray)⁷²⁻⁷⁶. GCK-III subfamily members have a short unconserved C-terminal stretch⁷⁷⁻⁷⁹. GCK-VI subfamily members are structurally reminiscent of those of GCK-II and GCK-III, but they have the conserved short C-terminal region of unknown function (light blue)⁸⁰⁻⁸². In the GCK-V subfamily, there is a less-conserved intermediate region, and a C-terminal AT1-46 homology domain (ATH, purple)^{40,41,83}. *Drosophila* NINAC, a kinase essential for phototransduction, has a Ste20-like kinase domain fused with a myosin head domain (light purple) and a calmodulin-binding domain (green)⁵⁹. The recent discovery of human class III myosin, an ortholog of NINAC⁶¹, has given rise to the GCK-VII subfamily which does not have a *Caenorhabditis elegans* ortholog. SNICK is a putative kinase predicted from human genome sequence (I. Dan *et al.*, unpublished). Finally, the GCK-VIII subfamily members contain a short less-conserved intermediate region, a long conserved region (dark green), and a C-terminal less-conserved stretch^{30-32,34}. They are integrated into the GCK family because of the location of the kinase domain, but might form a distinct family. Putative kinases CG14895 in *Drosophila* and Y38F1A.10 in *C. elegans* are not incorporated into any subfamily because they do not have a mammalian ortholog.

Epistatic analysis locates Ste20p directly upstream of Ste11p⁶, but this does not mean that Ste20p takes an integral part in the linear sequential phosphorylation cascade. A physically more suitable interpretation is that the two different signaling modules meet on the plasma membrane following stimulation by a pheromone, and transmit a signal by phosphorylation⁹⁻¹¹ (Fig. 1a). Ste20p

Box 2. Confusion in the nomenclature of PAKs

Unlike the varied names of germinal center kinase (GCK)-family kinases, human p21-activated kinase (PAK)-family kinases have been numbered sequentially^{a,b}. However, two major misunderstandings are prevalent: (1) the PAK that phosphorylates Raf-1 was called PAK3 in some papers^{c,d} but was later found to be PAK2^a, and (2) a human 'PAK2' on the DDBJ/EMBL/GeneBank database (AF092132) is a chimeric clone of PAK3, 1 and 2 from N-terminal to C-terminal.

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indeed acts as an MAP4K, but should not be included in the conserved MAPK signaling module. Rather, Ste20p should be regarded as one of the upstream activators of the MAPK pathways.

PAKs and GCKs are the mammalian homologs of Ste20p

In mammals there are two families of kinases related to Ste20p: the PAK and the GCK families (Box 1)^{1–3}. They can be distinguished by the location of their kinase domains: those of Ste20p and the PAKs are located at their C-termini, whereas those of the GCKs are at their N-termini. The structure of the kinase domain is conserved among Ste20p, PAKs and GCKs. A kinase domain is subdivided into 11 conserved subdomains¹². In the subdomain VIII of Ste20 group kinases, there is a distinct peptide sequence, (v/i)GTPyWMAPEv (a small letter indicates less conservation), termed the Ste20 signature sequence². Because the subdomain VIII is mainly responsible for substrate recognition in many kinases (possibly including Ste20 group kinases)¹², it is expected that the Ste20 group kinases have similar substrate selectivities. It follows that the mammalian Ste20 group kinases might phosphorylate MAP3Ks in the same way that the yeast Ste20p phosphorylates the yeast MAP3K. This is one of the primary reasons why the mammalian Ste20 group kinases have been reputed to be MAP4Ks.

Phylogenetic analysis of the human Ste20 group kinases
So far six human PAK and 22 human GCK family members have been cloned. Progress in the human genome project might result in finding of a few more

genes, but our extensive analysis of the EST and genomic databases predicts that the chances of finding more genes are slim. Thus the final number of Ste20 group kinases is likely to be around 30 in humans. These kinases were classified into subfamilies based on the extent of conservation of the kinase domain, as shown in Fig. 2. The general structural features of each subfamily are also illustrated in Fig. 2. The PAK and GCK families can be divided into two and eight distinct subfamilies, respectively. Phylogenetic analysis based on the structure of the noncatalytic region resulted in a similar classification, demonstrating that the kinases in each subfamily have distinct structural features in the noncatalytic regions and in the kinase domains.

Historically, the mammalian Ste20 group kinases have been classified into three families, PAKs, GCK-I and GCK-II^{1–3}, but our phylogenetic analysis has revealed more diversity. The revised classification is given support because the kinases in each subfamily share structural similarities within and outside the kinase domain, and because when the *Drosophila* and *Caenorhabditis elegans* homologs are incorporated into the analysis, each human subfamily is represented by a distinct *Drosophila* or *C. elegans* ortholog (except for GCK-VII without a *C. elegans* ortholog). This suggests that the structural prototype of each subfamily kinase was established during the early stages in the history of multicellular organisms. As a result of domain shuffling during evolution, the Ste20-like kinase domain might have fused with other functional domains to produce ancestral kinases of each mammalian subfamily. Their basic structures might have been established before the emergence of nematodes and then passed on to organisms such as flies and mammals. The number of human homologs (2–4) in each subfamily is in accordance with a putative two rounds of whole genome duplication in early vertebrates¹³.

Mammalian Ste20 group kinases that might act as MAP4Ks

HPK1 phosphorylates MEKK1 and MLK3 in vitro, whereas PAK2 phosphorylates Raf-1 both in vitro and in vivo
Three mammalian Ste20-like kinases have been shown to phosphorylate MAP3Ks. Hematopoietic progenitor kinase (HPK1), which belongs to the GCK-I subfamily, directly phosphorylates MAPK kinase kinase 1 (MEKK1) and mixed-lineage kinase 3 (MLK3) *in vitro*^{14,15}. HPK1 predominantly activates the stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) pathway^{14,15}. The activation of SAPK by overexpressed HPK1 is inhibited by the dominant-negative form of MEKK1, MLK3 and MKK4 (MAP2K), whereas the kinase-deficient form of HPK1 cannot impair the activation of SAPK by MLK3^{14,15}. Thus the sequential phosphorylation pathway HPK1–MEKK1/MLK3–MKK4–SAPK is suggested^{14,15} (Fig. 1c). These

studies provided the first experimental evidence for the existence of MAP4K activities in mammalian cells. The only other GCK family kinase that has been shown to phosphorylate MAP3K is GCK-like kinase (GLK)¹⁶.

In the MAPK pathway activated by the GTPase Ras, PAK2 is reported to activate the MAP3K Raf-1 by direct phosphorylation¹⁷ (Fig. 1b). PAK2 (mistakenly identified as PAK3, see Box 2), which is activated by the binding of the small GTPase Rac1 or Cdc42, phosphorylates Raf-1 at Ser338 both *in vivo* and *in vitro*, and overexpression of the kinase-defective PAK2 inhibits the activation of Raf-1 by Ras¹⁸. The Raf-1 mutant defective for Ras binding can still be activated by an active form of Ras, and the activation is blocked by a kinase-defective form of PAK2¹⁸. Moreover, the Ser338 phosphorylation of Raf-1 induced by epidermal growth factor (EGF) stimulation is impaired by an inhibitor of phosphoinositide 3-kinase (PI 3-kinase)¹⁷ suggesting that PAK2-mediated Raf-1 activation by Ras involves PI 3-kinase¹⁷.

Active PAK1 (structurally very similar to PAK2) also phosphorylates MEK1, a mammalian MAP2K, at Ser298, which is important for the stable binding of MEK1 and Raf-1¹⁹. This appears to involve MAP3K activity. However, PAK1 should not be regarded as a part of the triple-kinase module of the MAPK signaling cascades. As in the case with yeast Ste20, the more appropriate view might be an upstream signaling complex containing PAK-I activating a downstream MAPK signaling module (Fig. 1b). This activation might include what appears to be MAP4K activity as in the case of PAK2, and MAP3K activity as in the case of PAK1.

The binding of GCKs rather than their kinase activities might be important for activation of MAP3Ks

GCK-I subfamily members other than HPK1 are thought to mediate the tumor necrosis factor α (TNF α) signaling to the SAPK pathway³. However, SAPK activation by GCK-I kinases occurs largely because of their binding to MAP3Ks rather than phosphorylating them. When GCK-IIs are overexpressed in cells, they constitutively activate the SAPK pathway (but not the ERK or p38 pathway)³, but even forms of GCK and GLK lacking a kinase domain are capable of activating the SAPK pathway (albeit more weakly), suggesting that this activation is mediated by binding to MEKK1^{16,20,21} (Fig. 1d). Another possibility is that the activation is due to the formation of homopolymers of the mutant kinases and their endogenous wild-type forms. Similar activation of SAPK by kinase-defective forms is also observed for GCK-IVs such as NIK/HGK (partial activation), TNIK and MINK (full activation)²²⁻²⁴.

Several recent studies on MAP3Ks help to elucidate the kinase-independent activation of MAP3Ks by putative MAP4Ks. MLK3 forms

homodimers mediated by its leucine zippers, and their subsequent intermolecular autophosphorylation is sufficient for the activation of MLK3, which leads to the activation of SAPK²⁵. TAK1, an MAP3K that acts downstream of HPK1 and NIK/HGK, is activated by intramolecular autophosphorylation following binding of the cofactor TAB1^{26,27}. (The distinction between intramolecular and intermolecular autophosphorylation can be made by examining the phosphorylation of kinase-defective mutants in the presence and absence of the wild-type kinase.) The overexpression of TAK1 and TAB1 is sufficient to activate SAPK and p38 MAPK²⁷. MEKK1, a downstream MAP3K candidate for all mammalian GCK-I and GCK-IV kinases, is also activated by intramolecular autophosphorylation^{28,29}. These results indicate that mechanisms that foster the dimerization of MAP3Ks or stabilize MAP3K structures to allow intramolecular phosphorylation are important for activation of MAP3Ks, suggesting that putative MAP4Ks are responsible for stabilizing the structures of the activated MAP3Ks.

The role of the catalytic activity of putative MAP4Ks remains obscure in most cases, but it might be used for autophosphorylation to enhance their binding affinity to MAP3Ks. The kinase-defective form of GCK binds to MEKK1 less strongly than does wild-type GCK, correlating with a lesser degree of activation of SAPK by the kinase-dead GCK²¹. The strongest binding and SAPK activation is achieved by the free noncatalytic domain²¹. Therefore, autophosphorylation of GCK is likely to change its intramolecular conformation so as to make its noncatalytic region more accessible to MEKK1²¹ (Fig. 1c).

Various models should be tested to elucidate the mechanism of activation of putative MAP4Ks (two of them are shown in Fig. 1) as their phosphorylation substrates have not been examined extensively. Meanwhile, the presence of other pathways for activation of MAP3Ks *in vivo* should be kept in mind.

GCK-VIIIs are not MAP4Ks at all

TAO1 and PSK1/TAO2 in the GCK-VIII subfamily act as genuine MAP3Ks in the conserved triple-kinase MAPK signaling modules³⁰⁻³². TAO1 phosphorylates MKK3, which in turn specifically activates the p38 MAPK pathway³¹. Similarly, TAO2 phosphorylates MKK3 and MKK6, which are the specific activators of p38 MAPK³². PSK1, a human homolog of TAO2, phosphorylates MKK4 and MKK7 (indicating that it is an MAP3K) and exclusively activates the SAPK pathway³⁰. By contrast, JIK, the other mammalian kinase in the GCK-VIII subfamily, has not been demonstrated to be an MAP3K, but probably acts as a negative regulator in the SAPK pathway because JIK activation downregulates the activation of SAPK after EGF stimulation^{33,34}. Currently, GCK-VIII subfamily

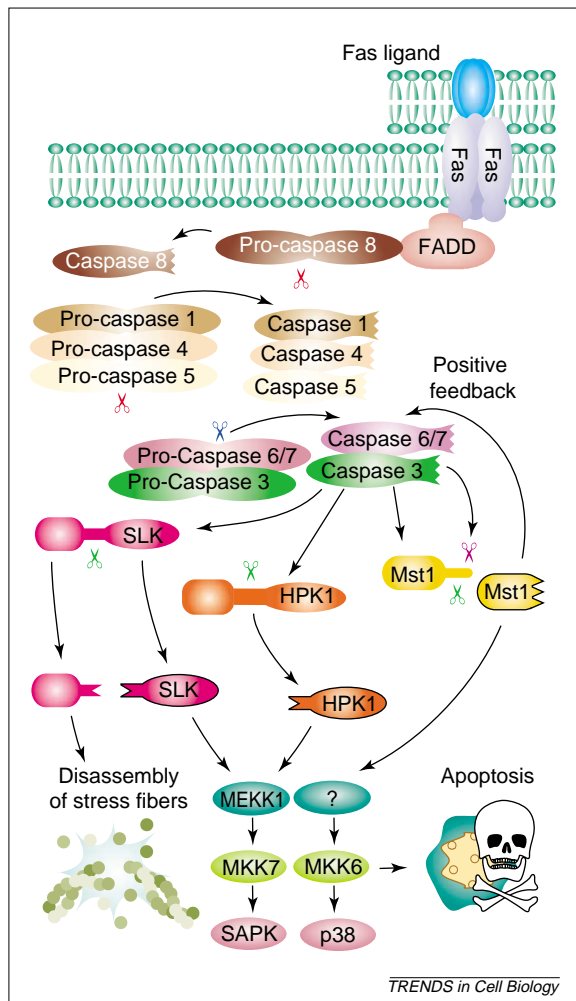


Fig. 3. Involvement of Ste20 group kinases in Fas-induced apoptosis. Following the binding of the Fas ligand, Fas recruits FADD and pro-caspase 8, which is cleaved to yield caspase 8³⁵ (red scissors, indicating caspase cleavage site). Caspase 8, in turn, cleaves pro-caspase 1, 4 and 5³⁵ (red scissors). The activated caspases finally cleave pro-caspase 3, 6 and 7 to produce caspase 3, 6 and 7^{35,42}, which in turn cleaves various Ste20 group kinases including Mst1, HPK1, SLK and PAK2 (green and purple scissors, PAK2 is not shown)³⁶⁻⁴². Mst1 activates the caspases that lead to cleavage of Mst1 itself, forming a positive feedback loop^{37,38}. Mst1 is cleaved by either or all of caspase 3 (green scissors) and caspase 6 and 7 (purple scissors) at different sites depending on cell conditions^{37,38,42}. The cleaved active kinase domains activate the SAPK pathway (MEKK1-MKK7-SAPK) and the p38 MAPK pathway (unidentified MAP3K-MKK6-p38 MAPK) to promote apoptosis^{37,38,42}. The caspase cleavage of HPK1 releases the activated kinase domain, resulting in the induction of apoptosis³⁹. SLK is cleaved to produce the activated kinase domain that induces apoptosis and a noncatalytic region that fosters disassembly of stress fibers^{40,41}.

members are incorporated into the GCK family, but further characterizations might allow them to form a new kinase family in the Ste20 group (Box 1).

The definition of MAP4K

The term MAP4K has already appeared in the literature and public databases and the human uni-gene organization (HUGO) has recently adopted MAP4K as an official classification. However, the definition of MAP4K is yet to be established. As in the cases of MAPK, MAP2K and MAP3K, the simple

definition of MAP4K would be a kinase that activates one of the MAPK cascades by directly phosphorylating MAP3K. Because the mechanism of MAP3K activation by the upstream kinases could be achieved without phosphorylation, caution is required in using such a classification. At present, use of a looser definition, such as a kinase that is involved in activating one of the MAPK pathways by interacting with MAP3K (without any evidence of direct phosphorylation), is prevalent. Under this definition, PAK-I, GCK-I and GCK-IV subfamilies can all be categorized as MAP4Ks. At the very least, however, evidence for direct phosphorylation of MAP3K should be required for a kinase to be known as an MAP4K.

Several Ste20 group kinases are involved in apoptosis. The involvement of the mammalian Ste20 homologs in the intracellular signaling pathways is not limited to their function as upstream activators of an MAPK pathway (including potential functions as MAP4Ks) and their functions should not be oversimplified. Many of the Ste20 homologs are used in signaling events, including apoptosis, morphogenesis and cytoskeletal rearrangement including cell motility and cell-shape changes (such as axon targeting).

Several Ste20 group kinases from different subfamilies are involved in inducing or preventing apoptosis, some activate MAPK pathways during apoptosis, whereas others regulate cellular events induced during apoptosis. In a typical apoptotic pathway (Fig. 3), activation of Fas by ligand binding induces a sequential cleavage of caspases³⁵ (red scissors in Fig. 3). The activated caspases finally cleave the most downstream effector pro-caspases³⁵ (blue scissors in Fig. 3) to produce activated effector caspases. One of the activated effector caspases, caspase 3, in turn cleaves various Ste20 group kinases including Mst1, Mst2, HPK1, SLK and PAK2³⁶⁻⁴¹ (green scissors in Fig. 3).

Mst1 can induce apoptosis when overexpressed in cells, which involves a positive-feedback loop consisting of two phases: Mst1 first activates the caspases, which in turn activate caspase 3^{37,38}. Then caspase 3 cleaves Mst1 at the DEMD³²⁶S caspase 3 recognition site (green scissors in Fig. 3), releasing the C-terminal regulatory region, which includes the inhibitory and dimerization domains, to produce a more active truncated form of Mst1^{37,38} (Fig. 3, right-hand side). Its kinase activity is essential for both intact and truncated forms to induce apoptosis, and also for the activation of SAPK and p38 MAPK^{37,38}. Mst1 and 2 were thought to act equally because of a high degree of sequence conservation³⁷, but a recent study has detected the second caspase cleavage site that is only present in Mst1⁴². The TMTD³⁴⁹G motif is recognized by caspases 6 and 7 (purple scissors in Fig. 3) and is resistant to caspase 3⁴². The caspase 3 recognition site is more susceptible to Fas-ligand

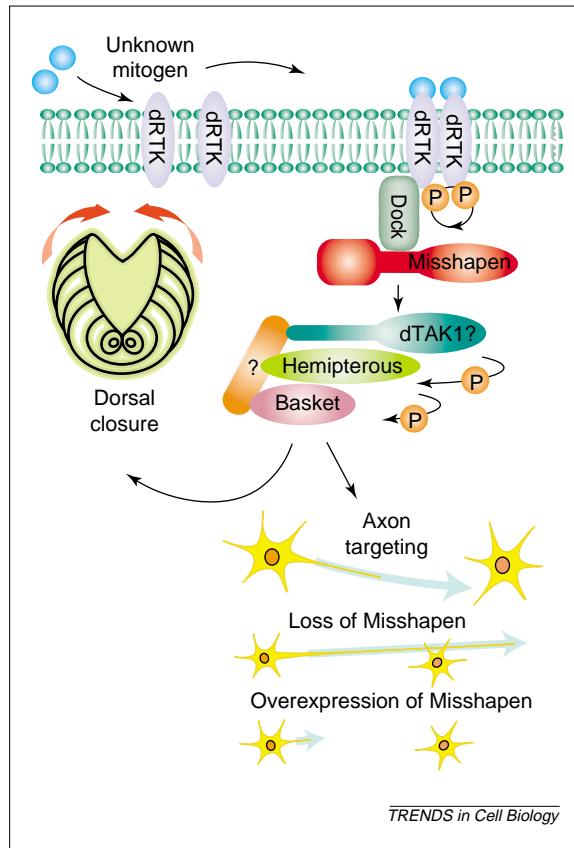


Fig. 4. Signaling through Misshapen. *Drosophila* Misshapen regulates dorsal closure and axon targeting^{52–55}. Upon binding of an unidentified mitogen, putative Eph-like receptor tyrosine kinases (RTKs) can be activated and recruit the *Drosophila* Nck homolog adaptor protein Dock, which binds to Misshapen^{51–53}. This leads to the activation of a triple-kinase signaling module probably comprising an unidentified *Drosophila* MAP3K (probably dTAK1), the *Drosophila* MAP2K hemipterous, the *Drosophila* SAPK basket, and an unidentified scaffold protein^{56,57}. The formation of the Misshapen–Dock complex is essential for the change in cell shape and motility during dorsal closure, and for regulation of axon targeting^{52,54,55}. These events are likely to be regulated through SAPK activation^{52,54–57}. Overexpression of Misshapen results in the pretarget growth-cone termination, whereas the loss of Misshapen induces overprojection of the growth cone^{54,55}.

treatment in BJAB cells, whereas that of caspase 6/7 is susceptible to interleukin 2 (IL-2) withdrawal in CTLL-2 cells⁴². This suggests that Mst1 and 2 regulate apoptotic events by different mechanisms. In addition, epistasis analysis using the dominant-negative form of MEKK1 (without direct phosphorylation analysis) locates Mst1 upstream of MEKK1, and also of MKK7⁴². MEKK1 undergoes caspase 3 cleavage during apoptosis⁴³, but how the MAPK signaling module is organized in this process remains uncertain (only linear cascades are shown in Fig. 3).

Other GCKs such as HPK1³⁹ and SLK^{40,41} are also activated during apoptosis. During Fas-induced apoptosis, caspase 3 cleaves HPK1 at Asp385, releasing the N-terminal kinase domain, which has an enhanced potential to activate SAPK³⁹ (Fig. 3, centre). SLK is cleaved by caspase 3 at the N-terminal region of the M-NAP domain to produce

two functionally active peptides^{40,41}. The N-terminal kinase fragment promotes apoptosis, whereas the C-terminal fragment promotes the disassembly of stress fibers⁴¹ (Fig. 3, left-hand side).

PAK2 (but not PAK1 and probably not PAK3 which does not have caspase 3 target motifs) is activated by caspase cleavage during Fas- and TNF α -induced apoptosis, and the cleaved C-terminal kinase domain induces formation of apoptotic bodies³⁶. On the other hand, PAK1 has been shown to promote cell survival during apoptosis induced by IL-3 depletion⁴⁴. During this process, anti-apoptotic proteins Bcl-2 and Bcl-x_L are inactivated by hetero-oligomerization with the pro-apoptotic protein Bad⁴⁴. Phosphorylation of Bad by PAK1 following IL-3 treatment results in dissociation of Bad from Bcl-2 and Bcl-x_L, leading to cell survival⁴⁴. Moreover, PAK1 acts downstream to Akt, an effector kinase of Ras and PI 3-kinase, to promote cell survival⁴⁵. It seems strange that structurally similar kinases in the same subfamily can be both pro- and anti-apoptotic. Even more contradictory, PAK2 itself acts against apoptosis in BALB3T3 cells treated with TNF α , growth factor withdrawal, and UV light in a similar mechanism to PAK1⁴⁶. One possible explanation for the contradictory action of PAK2 is that different apoptotic pathways might use PAK2 in different ways depending on cell types and conditions. The anti-apoptotic activity of PAK1 and 2 might be more widely shared by other PAK family members. PAK4 in the PAK-II subfamily can also prevent apoptosis by promoting phosphorylation of Bad⁴⁷.

Several Ste20 group kinases affect morphogenesis and the cytoskeletal rearrangement

Regulation of the cytoskeleton is a recurring theme in the function of Ste20 group kinases. Because the role of PAK-I subfamily kinases in the regulation of the actin cytoskeleton has recently been reviewed^{1,2}, we will concentrate on the function of the PAK-II, GCK-IV, GCK-VII and GCK-VIII subfamilies.

PAK-II subfamily kinases are probably involved in the reorganization of the actin cytoskeleton but in different ways from those of PAK-I subfamily kinases⁴⁸. The activity of the PAK-II family might not be dependent on the Rho-family small GTPases, unlike that of PAK-I⁴⁸. For example, Cdc42 can bind to PAK4 (in the PAK-II subfamily) but is involved in the translocation of PAK4 to the Golgi apparatus rather than in its activation⁴⁸. PAK4 can induce filopodia formation through the rearrangement of the cytoskeleton but its regulatory mechanism remains uncertain⁴⁸. PAK-II subfamily members might play integral roles in neuronal morphogenesis as a deficiency of PAK-II (mbt gene product) in *Drosophila* results in severe defects in central brain structure⁴⁹. A different role has been assigned to recently cloned PAK6 despite its structural similarity to other PAK-II kinases. It is

preferentially expressed in testis and binds to the androgen receptor⁵⁰. In response to androgen, it translocates to the nucleus resulting in repression of androgen receptor-mediated transcription⁵⁰.

PSK1, a member of the GCK-VIII subfamily acting as an MAP3K, is localized on cytoplasmic vesicles when microinjected and induces the break up of actin stress fibers³⁰. The noncatalytic region alone can induce such disassembly of the cytoskeleton³⁰. This study shows the bifunctionality of PSK1 in activating SAPK (through the kinase domain phosphorylating MKK4 and 7) and regulating the actin cytoskeleton (through the noncatalytic domain)³⁰.

GCK-IV family kinases NIK and TNIK bind to an Src-homology 3 (SH3)-containing adaptor protein, Nck, at the conserved proline-rich motif^{22,23}. Nck can interact with a variety of molecules including those involved in cytoskeletal regulation such as PAK-I family kinases and the Wiskott–Aldrich syndrome protein (WASP)⁵¹. Misshapen, a GCK-IV ortholog in *Drosophila*, provides an interesting example (Fig. 4). It is upstream of the *Drosophila* SAPK basket, and of MAP2K hemipterous, whereas it is downstream of the *Drosophila* Nck homolog Dock. Dock is thought to associate with an unknown Eph-like receptor tyrosine kinase because Dock is similar enough to Nck and can bind to the vertebrate Eph receptor tyrosine kinases EphB1 and B2, as does Nck^{52,53}. Both Misshapen and Dock are required for dorsal closure in late *Drosophila* embryogenesis, and for axon guidance^{52,54,55} (Fig. 4). In the *Drosophila* visual system, overexpression of Misshapen resulted in the termination of growth-cone to the target cells, whereas the loss of Misshapen induced projection of the growth cone past its target⁵⁴. The MAP3K component in this pathway remains uncertain, but it is likely to be dTAK1 because loss of dTAK1 results in the same dorsal-open phenotype as Dock, Misshapen, basket and hemipterous mutant^{56,57}. In *C. elegans*, MIG15 (a GCK-IV ortholog) is essential for the migration of central nerve cells⁵².

Another line of evidence, however, shows that the defect in axon targeting in the *Drosophila* visual system in the loss-of-function mutant of DOCK is indistinguishable from that of *Drosophila* PAK-I, DPAK⁵⁸. Because overexpression of the gain-of-function type of DPAK in the dock-deficient mutant can rescue the defective axon-targeting phenotype, DPAK also acts as a DOCK effector⁵⁸. It is possible that DOCK–DPAK and DOCK–Misshapen interactions work in concert to regulate axon guidance. If both result in activation of the SAPK, however, it might be possible that either of the overexpression rescue experiments represents an experimental artifact. Hence, examination of the downstream effectors is important.

GCK-VII subfamily members are likely to be integral players in the phototransduction process in

the retina. Most knowledge of GCK-VII has been derived from the study of *Drosophila* NINAC. NINAC has two splice variants: a shorter p132 with a calmodulin-binding site at the end of the myosin head domain, and a longer p174 with an additional calmodulin-binding site and a binding domain with INAD, an adaptor protein with five PDZ domains that interacts with calmodulin, phospholipase C, protein kinase C and several transmembrane proteins including rhodopsin⁵⁹. The binding of NINAC p174 to INAD is essential for the localization of NINAC in the microvilli of photoreceptor cells (rhabdomeres) and for termination of the photoresponse⁶⁰. The role of the kinase domain of NINAC is not clear except for its autophosphorylation activity *in vitro*⁵⁹. Like other myosins, NINAC cosediments with F-actin, and the binding of ATP disrupts this interaction⁵⁹. Loss of the myosin head domain or the calmodulin-binding domain of NINAC results in fragmentation of the rhabdomeres⁵⁹. These findings will be valuable for understanding the functions of newly found mammalian NINAC homologs⁶¹ (I. Dan *et al.*, unpublished).

Perspectives

A notable feature of the Ste20 group kinases is their extensive structural diversity in their noncatalytic domains, in contrast with the conservation occurring within the kinase domains. Such variations enable these kinases to be involved in a variety of intracellular signaling pathways. Most kinases in this group modulate the signaling of MAPK pathways at various epistatic stages, and some appear to have putative functions as MAP4Ks. However, integration of Ste20 group kinases into a simplistic linear kinase activation cascade should be avoided even if some of them do phosphorylate MAP3Ks. A proteomic paradigmatic rather than a linear genetic view has been suggested to understand the function of Ste20 group kinases⁶². Acting as major players in upstream signaling complexes, Ste20s activates downstream MAPK signaling modules, and might add specificity to the conserved MAPK signaling pathway to participate in cellular phenomena such as apoptosis, morphogenesis and rearrangement of the cytoskeleton.

On the basis of this newly established phylogenetic relationship, a more systematic understanding of the very diverse functions of the Ste20 group kinases can be gained. It is safe, however, to assume that the kinases belonging to the same subfamily always have similar functions. For example, motifs in unconserved regions (e.g. the caspase 3 cleavage site in PAK2, HPK1, Mst1/2, and SLK) also confer various physiological functions on the kinases in the same subfamily.

Yeast has served as a good model for elucidating the basic MAPK signaling cascades. For Ste20 group kinases, however, yeast can only serve as a model for

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the PAK-I subfamily members since other members of the mammalian Ste20 group have probably emerged after the divergence of yeast from other eukaryotes. The phylogenetic relationship proposed in this review suggests that ancestors of the mammalian Ste20 family kinases were established before the emergence of nematodes, conserved in flies and mammals, and then branched further to yield more diversity in mammals. In this sense, *Drosophila* and *C. elegans* should serve as useful systems in which each subfamily is represented by a single kinase, and thus the interference from other kinases in the same

subfamily can be avoided. This is exemplified by the *Drosophila* Misshapen^{52,54,55} and NINAC^{59,60}, but most of the ancestor kinases have remained unexamined. Given the recent progress in reverse genetics, these *Drosophila* and *C. elegans* orthologs should be exploited to elucidate the characteristic functions of each mammalian subfamily. Knowledge obtained from different species based on the correct phylogenetic relationships would contribute significantly to elucidating the functions of the mammalian Ste20 group kinases which we are only just beginning to understand.

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