Signaling by the Germinal Center Kinase Family of Protein Kinases*

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Mammalian mitogen-activated protein kinase (MAPK) pathways regulate an extensive range of cellular processes including gene transcription, cytoskeletal organization, metabolite homeostasis, cell growth, and apoptosis. At the physiologic level, MAPK pathways are likely to be critical to the pathogenesis of a number of important clinical conditions including oncogenesis, diabetes, ischemic injury, arthritis, and septic shock. MAPK pathways have been widely conserved in eukaryotic cell evolution. At the heart of these pathways are so-called "core signaling modules" consisting of the MAPKs, which are activated by concomitant Tyr and Thr phosphorylation catalyzed by members of the MAPK/extracellular signal-regulated kinase (ERK) kinase (MEK) family. MEKs, in turn, are activated by Ser/Thr phosphorylation catalyzed by protein kinases of several families collectively termed MAP kinase kinases (MAP3Ks) (reviewed in Refs. 1–3). Mammalian cells possess at least six MAPK families, three of which have been characterized in some detail: the ERKs, the stress-activated protein kinases (SAPKs, also referred to as Jun N-terminal kinases or JNKs) and the p38s (1, 2). The ERK pathway is a major downstream target of the Ras proto-oncoprotein and has been reviewed extensively elsewhere (2, 4). The SAPKs and p38s are, in most instances, poorly activated by mitogens and are instead potently and preferentially activated by a variety of environmental stresses (ionizing radiation, heat shock, oxidative stress, osmotic shock), inflammatory mediators of the TNF family (TNF, interleukin-1, CD40L, etc.), and the vascular responses to ischemia, reperfusion, and hypertension and associated humoral factors (interleukin-1, CD40L, etc.), and the vascular responses to ischemia, reperfusion, and hypertension and associated humoral factors (interleukin-1, CD40L, etc.), and the vascular responses to ischemia, reperfusion, and hypertension and associated humoral factors (interleukin-1, CD40L, etc.), and the vascular responses to ischemia, reperfusion, and hypertension and associated humoral factors (interleukin-1, CD40L, etc.). The SAPKs and p38s activate several transcription factors, most notably activator protein-1 (reviewed in Refs. 1 and 5).

The SAPKs are activated by at least two MEKs, SAPK/ERK-kinase-1 (also called MAPK kinase (MKK)-4) and MKK7. The p38s are also activated by at least two MEKs, MKK3 and MKK6 (6–10). The MAP3Ks upstream of the SAPKs and p38s are structurally divergent and differ widely in the spectrum of MEKs that they can activate in vivo and in vitro. Of these, only MEK kinase 1 (MEKK1) and mixed lineage kinases (MLK) 2 and 3 are demonstrably SAPK pathway-specific (11–20) (reviewed in Ref. 1).

Although considerable progress has been made in the identification of the molecular components and regulatory relationships of which MAPK core signaling modules are composed, much less is known of how core signaling modules are linked to events at the cell surface. A bewildering array of potential upstream activating proteins has been implicated in the regulation of MAP3Ks, ranging from Ras superfamily GTPases to additional protein kinases and adapter proteins coupled to cytokine receptors. In particular, the SAPKs and p38s can be activated in vivo by Rac1, Cdc42Hs, and V12 Chp, members of the Rho subgroup of Ras family GTPases (21–24). Most, but not all, Rac and Cdc42 effectors possess a Cdc42/Rac interaction and binding (CRIB) domain (25, 26). Of note, p21-activated kinases (PAKs) possess CRIB motifs and are activated upon binding GTP-Rac1 or -Cdc42Hs (27). Several MAP3Ks upstream of the SAPKs and p38s, including MEKK-1 and -4 and MLK-2 and -3, can also bind GTP-Rac1 and/or -Cdc42Hs.

Recently, protein Ser/Thr kinases related to human germinal center kinase (GCK) have emerged as important potential players in the regulation of stress-activated MAPK core signaling pathways. This review will discuss what is known about the GCKs and their roles in MAPK pathway regulation.

The GCK Family: Structural Features of Group I and II Enzymes

Eleven mammalian protein kinases related to GCK have been cloned. In addition, there are Drosophila, Caenorhabditis elegans, and Dictyostelium homologues as well as two Saccharomyces cer- evisiae genes with defined phenotypes. All GCK homologues possess N-terminal kinase domains that are distantly related to those of the PAKs and extensive C-terminal regulatory domains (CTDs) (30–44) (Fig. 1). The distant homology between PAK and GCK kinase domains has led to the grouping of these kinases into a single family. However, GCKs do not possess CRIB motifs and do not bind Rho GTPases; moreover, the PAKs have C-terminal kinase domains (27). Based on these strong differences, GCKs should be considered a distinct protein kinase family.

GCKs can be subdivided into two broad groups based on their structural and functional properties. Group I GCKs are closely related to GCK itself and include GCK, GCK-related (GCKR), GCK-like kinase (GLK), hematopoietic progenitor kinase-1 (HPK1), Nck-interacting kinase (NIK, not to be confused with NF-κB-inducing kinase, also called NIK), and Drosophila Misshapen. These enzymes have been shown to activate selectively the SAPKs (30–35, 41). The C. elegans GCK MIG-15, an ortholog of NIK, is also a group I GCK (Fig. 1).

The C-terminal domains of all group I GCKs include at least two proline/glutamic acid/serine/threonine (Pest) motifs and at least two polypeptide consensus binding sites for proteins containing Src homology (SH)-3 domains (30–35, 41). Most significantly, however, all of the group I kinases possess a highly conserved ~350-aa C-terminal region divided into two domains: a hydrophobic, leucine-rich domain and a 140–150-aa stretch, the C-terminal (CT) region (Fig. 1) (30–35, 41). The leucine residues in the Leu-rich domains are not organized into leucine zippers nor are these domains sufficiently hydrophobic for membrane insertion (Fig. 1) (30–35). GCK, GCKR, and GLK are all activated in vivo by TNF, and their CTDs along with that of HPK1 are quite homologous. Similarity to the CT motif of NIK, although apparent, is less dramatic (30–32, 44). Studies of GCK and GCKR indicate that this domain is required for binding proteins of the TNF receptor-associated factor (TRAF) family and, possibly, for gating the binding of MAP3Ks (45). Group II GCKs (Ste20-like oxidant stress-activated kinase-1

* This minireview will be reprinted in the 1999 Minireview Compendium, which will be available in December, 1999. Work in the author's laboratory is supported by United States Public Health Service Grant GM46577 and a basic science grant from the Arthritis Foundation.

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1 The abbreviations used are: MAPK, mitogen-activated protein kinase; aa, amino acids(s); CD, cluster of differentiation; Cdc, cell division cycle; CRIB, Cdc42/Rac interaction and binding; CT, GCK C-terminal extension of CTD; CTD, C-terminal regulatory domain; ERK, extracellular signal-regulated kinase; GCK, germinal center kinase; GCKR, GCK-related; GLK, GCK-like kinase; HPK1, hematopoietic progenitor kinase-1; IRS, Insulin receptor substrate; JNK, c-Jun N-terminal kinase; LOK, lymphocyte-activated kinase; MAPK, MAP kinase kinase; MEK, MAPK/ERK kinase; MKK, MAP kinase kinase; MLK, mixed lineage kinase; MST, mammalian sterile twenty-like; NIK, Nck-interacting kinase; NIK-like kinase; PAK, PAK, p21-activated kinase; PEST, Pro/Glu/Ser/Thr-rich; RING, really interesting new gene; SAPK, stress-activated protein kinase; SH, Src homology; SOK, Ste20-like oxidant stress response kinase; SPS, sorupulation-specific; TNF, tumor necrosis factor; TRAF, TNFR-associated factor.

2 J. Kehrl, personal communication.
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Interaction of GCK and Rab8

A yeast two-hybrid screen employing the monomeric GTPase Rab8 as a bait demonstrated that GCK CTD could bind Rab8 in vivo. This binding is apparently GTP-dependent inasmuch as

3 J. M. Kyriakis and T. Yuasa, unpublished observations.
GT-Pase-deficient forms of Rab8 strongly interact in vivo with GCK, whereas Rab8 mutants that cannot bind guanine nucleotides or exchange GDP for GTP do not associate in vivo with GCK (49). Rab family GT-Pases are members of the Ras superfamily and have been implicated in the regulation of vesicular trafficking. Rab8 regulates traffic between the trans-Golgi and the plasma membrane (50). Thus GCK may play an effector role in Rab8-regulated vesicle movement, or alternatively, Rab8 may target GCK to substrates proteins associated with the cytosolic leaflets of vesicle membranes destined for fusion with the plasma membrane.

### SH3-binding Sites of Group I GCKs and Their Interactions with SH3 Adapter Proteins

The SH3-binding sites of the CTDs of group I GCKs are likely to be important in the regulation and function of these proteins in vivo. GCK, GCKR, GLK, and HPK1 each have one SH3 binding site in their conserved Leu-rich regions (30–32, 34, 35). These SH3-binding sites may serve in effector binding; thus, the C-terminal Leu-rich domain SH3-binding site of HPK1 interacts with the MLK3 SH3 domain (31) (see above).

In addition, both HPK1 and NIK can interact with SH3 domain-containing adapter proteins that couple to Tyr kinases (33, 51). These interactions may allow for the recruitment of these GCKs to the membrane. Thus, NIK was cloned as an interactor with Nck, an adapter protein that couples receptor Tyr kinases to cytoskeletal and cell shape changes (33, 52). Interestingly, the Drosophila and C. elegans orthologs of NIK, Misshapen, and MLG-15, respectively, have been implicated in the regulation of embryogenic functions that involve cell elongation and migration (41) (see below). NIK and its orthologs may regulate cellular and developmental processes that involve changes in cell motility and morphology.

HPK1 not only interacts in vivo with MLK3, but it also binds the SH2/SH3 domain-containing adapter protein Grb2 (51). Grb2 is known for its ability to interact constitutively with mSOS, a Ras guanine nucleotide-exchange factor. Mitogen-induced receptor Tyr kinase autophosphorylation causes Grb2-mSOS to bind to Tyr(P) residues on the activated receptors. mSOS is thereby recruited to membranes where it can activate Ras (reviewed in Refs. 2 and 4). The C-terminal SH3 domain of Grb2 binds preferentially to the N-terminal two SH3-binding sites of HPK1 (51). As with the Grb2-mSOS interaction, the Grb2-HPK1 interaction is not altered by mitogen stimulation. However, epithelial growth factor treatment does stimulate the translocation of the Grb2-HPK1 complex to the autophosphorylated epidermal growth factor receptor at the membrane. These findings raise the intriguing possibility that membrane translocation of group I GCKs can be regulated by Tyr kinases (Fig. 2).

### GCK and GCKR as Effectors for TRAFs

Three group I GCKs, GCK, GCKR, and GLK, are activated in vivo by TNF and appear to be elements in TNF signaling pathways that activate the SAPKs (34, 35, 45). The TNF family comprises a large group of related inflammatory mediators that bind to a group of related receptors to initiate responses that are critical to acquired and innate immunity, as well as to the pathogenesis of a number of important clinical conditions including arthritis, sepsis, inflammatory bowel disease, and type 2 diabetes mellitus (reviewed in Ref. 53).

TNF binds to one of two receptors, the 55-kDa TNF receptor (TNFR)-1/CD120a or the 75-kDa TNFR2/CD120b. Neither TNFR possesses intrinsic enzymatic activity. TNFRs homotrimmerize upon binding ligand, an event that triggers the recruitment of downstream effectors. The intracellular extension of TNFR1 contains a death domain orthologous to other death domains, in a TNF-dependent manner, TNFR-associated death domain protein (TRADD), which, in turn, recruits TRAF2 (reviewed in Ref. 53).

TRAF2 is one of six known mammalian members of the TRAF family, each of which consists of C-terminal conserved TRAF domains, central zinc finger repeats, and with the exception of TRAF1, an N-terminal RING finger domain. The RING finger is critical for TRAF2 signaling to downstream effectors. The TRAF domains mediate the binding of TRAF proteins to their upstream activators and downstream targets. Transient overexpression of TRAF2, -5, and -6 can activate the SAPKs (reviewed in Ref. 53).

### Genetic Studies of Group I GCKs: Signaling by Misshapen

Recent studies of the Drosophila dorsal closure signaling pathway lend credence to the idea that group I GCKs couple receptors (notably TNFR family receptors) to MAP3Ks. Dorsal closure occurs late in Drosophila embryogenesis and is precipitated by cell migrations and shape changes that position and eventually fuse the lateral epidermal primordia over the aminoserosa. Dorsal closure requires basket, the Drosophila homologue of SAPK, hemipterous, a homologue of M KK7, and Drosophila Jun, Djun. This Drosophila SAPK pathway induces expression of deca peptaplegic, a transforming growth factor-β homologue that ultimately controls the tissue reorganization characteristic of dorsal closure (reviewed in Ref. 54).

The Misshapen polypeptide is strikingly similar to NIK, both within and outside of the catalytic domain. Deletion of misshapen is lethal and results in dorsal closure defects similar to those arising from defects in basket (41). Ecopic expression of misshapen rescues the dorsal closure defects of mutant or null embryos. Transient expression of misshapen in mammalian cells results in activation of coexpressed SAPK indicating that Misshapen may signal to Basket. Consistent with this, a significant percentage of either doubly heterozygous misshapen−/+, basket−/+ or misshapen−/+, hemipterous−/+ flies exhibits a dorsal open phenotype, with the severity of the phenotype correlating well with the strength of the basket or hemipterous allele. Moreover, constitutively active Djun rescues not only the basket phenotype but the misshapen phenotype as well when expressed in the corresponding mutant embryos (41).
Skolnik and colleagues have recently identified a Drosophila TRAF homolog that binds Misshapen in vitro. Like mutations in misshapen, mutations in the Drosophila TRAF also give rise to dorsal closure defects; however, placement of the Drosophila TRAF upstream of Misshapen awaits further epistasis studies.

**Group II GCKs: Activation by Extreme Environmental Stresses**

No known effectors have been identified for mammalian group II GCKs. Like group I GCKs, group II kinases are essentially ubiquitously expressed (with one exception, LOK, which is selectively expressed in lymphocytes) and possess significant basal activity when immunoprecipitated from endogenous sources or when overexpressed (36–39, 43, 44). However, Krs1, MST1/Krsk2, and SOK1 can be activated substantially in vivo by different environmental stresses. Krs1 and MST1/Krsk2 are activated by extreme heat shock and high concentrations of arsenite, staurosporine, and okadaic acid (37, 38). MST1/Krsk2 can also be activated in vitro by phosphatase 2A (37, 38). Thus these two group II GCKs may be activated by both phosphorylation and dephosphorylation. SOK1, as its name implies, is strongly activated by oxidative stress. SOK1 is also activated by ischemic injury and depletion of the cellular ATP pool. In all cases, SOK1 activation appears to require the generation of reactive oxygen intermediates as well as elevated levels of cytosolic free Ca²⁺ (36, 55). Stimuli that recruit LOK and MST3 are unknown (39, 40). Certain group II GCKs display enzymologic properties that may yield clues as to the mechanisms of regulation of the GCK family. Thus, SOK1 and MST3 autoactivate upon autophosphorylation in vitro, and both SOK1 and MST1/Krsk2 spontaneously homodimerize in vivo (36, 37, 39, 55). These results suggest that oligomerization and autophosphorylation may play a role in GCK family kinase activation.

**Conclusions and Perspectives**

The GCKs represent an emerging family of protein kinases that regulate eukaryotic stress responses. How might the available results be combined into a general model suitable for further experimental testing? Fig. 2 illustrates one way in which group I GCKs might mediate activation of MAPKs. In this model activated receptor Tyr kinases (through SH2/SH3 adapters) or cytokine receptors (through TRAFs) trigger the translation of GCKs to membrane-associated receptor complexes. This translocation may initiate activation of GCKs (perhaps by oligomerization or inhibition) and bring GCKs into close apposition with membrane-associated MAP3Ks, thereby promoting MAP3K binding and activation. Many MAP3Ks including MKK1 (an effector for GCK, NIK, and HKPK1) and MLK3 (an HKPK effector) themselves display a reversible, stimulus-induced membrane translocation, which is required for activation (1–4, 28, 29). Differential and selective signal-induced MAP3K and GCK translocation would permit the specific activation of discrete pools of MAP3Ks by specific GCKs. Clearly further study of this interesting family of protein kinases will be important to our understanding of how MAPK core signaling modules are coupled to their activators.

**Acknowledgments**—I thank Edward Skolnik and Melanie Cobb for providing results prior to publication and John Rehji and Thomas Force for collaborations and for providing results prior to publication.

**Note Added in Proof**—A portion of the studies of a Drosophila TRAF cited in the text as “E. Skolnik, personal communication” (Footnote 4) is now published (Liu, H., Su, Y.-C., Becker, E., Treisman, J., and Skolnik, E. Y. (1999) J. Biol. Chem. 274, 9–10).