Mammalian Mitogen-Activated Protein Kinase Signal Transduction Pathways Activated by Stress and Inflammation

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I. INTRODUCTION

Mitogen-activated protein kinase (MAPK) signal transduction pathways are among the most widespread mechanisms of eukaryotic cell regulation. All eukaryotic cells possess multiple MAPK pathways, each of which is preferentially recruited by distinct sets of stimuli, thereby allowing the cell to respond coordinately to multiple divergent inputs. Mammalian MAPK pathways can be activated by a wide variety of different stimuli acting through diverse receptor families, including hormones and growth factors that act through receptor tyrosine kinases [e.g., insulin, epidermal growth factor (EGF), platelet-derived growth factor (PDGF), and fibroblast growth factor (FGF)] or cytokine receptors (e.g., growth hormone) to vasoactive peptides acting through G protein-coupled, seven-transmembrane receptors (e.g., ANG II, endothelin), transforming growth factor (TGF)-β-related polypeptides, acting through Ser-Thr kinase receptors, inflammatory cytokines of the tumor necrosis factor (TNF) family and environmental stresses such as osmotic shock, ionizing radiation and ischemic injury. MAPK pathways, in turn, coordinate activation of gene transcription, protein synthesis, cell cycle machinery, cell death, and differentiation. Accordingly, these pathways exert a profound effect on cell physiology (120, 165, 195).

All MAPK pathways include central three-tiered “core signaling modules” (Fig. 1) in which MAPKs are activated by concomitant Tyr and Thr phosphorylation within a conserved Thr-X-Tyr motif in the activation loop of the kinase domain subdomain VIII. MAPK phosphorylation and activation are catalyzed by a family of dual specificity kinases referred to as MAPK/extracellular signal-regulated kinase (ERK)-kinases (MEKs or M KKs). MEKs, in turn, are regulated by Ser/Thr phosphorylation, also within a conserved motif in kinase domain subdomain VIII, catalyzed by any of several protein kinase families collectively referred to as MAPK-kinase-kinases (MAP3Ks). MAPK core signaling modules are themselves regulated by a wide variety of upstream activators and inhibitors (120, 165, 195).

The notion of multiple parallel MAPK signaling cascades was first appreciated from studies of simple eukaryotes such as the budding yeast Saccharomyces cerevisiae. To date, six S. cerevisiae MAPK signaling pathways have been identified. These have been reviewed...
elsewhere (120). The features of yeast MAPK pathways as well as early biochemical studies have revealed several common principles shared by all MAPK pathways.

1) MAPKs are proline-directed kinases; however, substrate selectivity is often conferred by specific MAPK docking sites present on physiological substrates. The proline-directed substrate specificity of the MAP kinases was established using peptide substrates corresponding to the sequences surrounding Thr 669 of the EGF receptor (Glu-Leu-Val-Glu-Pro-Leu-Thr-Pro-Ser-Gly-Glu-Ala-Pro-Asn-Gln-Ala-Leu-Leu-Arg) and the site on myelin basic protein (Ala-Pro-Arg-Thr-Pro-Gly-Gly-Arg) phosphorylated in vitro by the 42-kDa insulin- and mitogen-stimulated MAPK ERK-2. Systematic variation in the sequences surrounding the single Thr phosphorylation site in the EGFR peptide and the single Thr in the MBP peptide, verified the essential role of the proline at immediately COOH terminal to the phosphoacceptor site (50, 55, 84). However, this proline directness is not sufficient to account for the high degree of substrate selectivity manifested by different MAPK subgroups. The Michaelis constant (K_m) for native protein substrates is usually several orders of magnitude lower than the K_m for synthetic peptides corresponding in amino acid sequence to the region immediately surrounding the phosphorylation site on these substrates. It has become apparent that most, if not all, of the physiological substrates of the MAPKs possess specific binding sites, often at considerable distance from the phosphorylation site in the primary sequence, that allow for a strong interaction with select MAPK subfamilies to the exclusion of others (64, 144, 301, 351). In turn, MAPKs themselves possess complementary docking sites that interact with the MAPK binding domains on substrate proteins (143, 301). This confers a striking substrate specificity on a family of protein kinases with an otherwise apparently broad in vitro substrate profile, certainly as portrayed by synthetic peptides.

2) There are signaling components with more than one biological function/signaling components under multiple forms of regulation. Within MAPK core signaling modules, there are instances wherein individual elements can function promiscuously in several pathways. Conversely, MAPK pathway components are often subject to regulation by multiple inputs. For example, the S. cerevisiae MAP3K Ste11p functions as part of the mating pheromone response pathway and the osmosensing pathway (20, 242), while the mammalian MAP3K tumor progression locus-2 (Tpl-2) can activate the mitogenic ERK and three stress-activated MAPK pathways (264). Conversely, the yeast osmosensing MEK Pbs2p can be regulated by three MAP3Ks: Ssk2p, Ssk22p, and Ste11p while the mammalian stress-regulated MEK stress-activated protein kinase/ERK-kinase-1 (SEK1)/MAPK-kinase-4 (MKK4) is a putative substrate for at least 10 known MAP3Ks.

3) Pathway organization is mediated by scaffolding proteins. Given the extraordinary complexity and diversity of MAPK regulation and function, it is critical that the efficiency and selectivity of MAPK pathways be preserved. Scaffold proteins bind and sequester select MAPK pathway components, and thereby help to maintain pathway integrity and to permit the coordinated and efficient activation and function of MAPK components in response to specific types of stimuli (234). Some yeast signaling pathways include distinct scaffolding proteins that bind and segregate groups of signaling components. Alternatively, in some MAPK pathways, the signaling components themselves possess intrinsic scaffolding properties (see Fig. 7). Thus Ste5p of the yeast mating pheromone pathway is a scaffolding protein that selectively binds a MAP3K (Ste11p), a MEK (Ste7p), and a MAPK (Fus3p) and couples them to upstream activators. Thus, although Ste11p can function in both the mating and osmosensing pathways, it selectively activates different MEKs in each pathway: Ste7p for the mating pathway and Pbs2p for the osmosensing pathway, due in part to the fact that Ste5p maintains signaling pathway specificity by binding selectively Ste7p and not Pbs2p (120, 242).

Conversely, Pbs2p, in addition to serving as a MEK, has intrinsic scaffold properties, selectively binding Ste11p and the osmosensing MAPK Hog1p. Pbs2p does not bind Fus3p, and thus Pbs2p maintains signaling pathway integrity by interacting specifically with Hog1p and not with Fus3p or Kss1p (3, 11). Likewise, the mammalian scaffold proteins c-Jun NH2-terminal kinase (JNK) interacting proteins (JIPs)-1 and -2 and JNK/stress-activated protein kinase (SAPK)-associated protein-1 (JSP1/JIP3), like Ste5p, couple elements of different SAPK core signaling modules, while mammalian stress-activated protein kinase (SAPK)/ERK-kinase-1 (SEK1), like Pbs2p, possess both the properties of a protein kinase and a scaffold protein (72, 136, 155, 336, 342, 355).

4) MAP3K regulation is by membrane recruitment, oligomerization, and phosphorylation. MAP3K regulation represents the entry point to MAPK pathways and is accordingly complex. In addition to scaffold proteins mentioned above, actual activation of MAP3Ks has been shown to involve three key phenomena: recruitment to the membrane, typically mediated by an upstream activating protein; homologimerization, often within a multi-protein complex containing additional regulatory components; and phosphorylation by upstream kinases. The mitogenic MAP3K Raf is illustrative of all three properties. Thus it is recruited to the membrane by Ras (195), where it undergoes regulatory phosphorylation catalyzed in part by p21-activated kinase-3 (160) and oligomerization (190). All three events are necessary for activation (see sect. miA7). Stress-regulated MAP3Ks are likely similarly regulated.

A. General Considerations

The insulin/mitogen-regulated extracellular signal-regulated kinase (ERK) pathway was the first mammalian MAPK pathway to be identified. This pathway is largely regulated by the monomeric GTPase Ras which recruits MAP3Ks of the Raf family to activate two MEKs: MEK1 and MEK2. These, in turn, activate the ERKs. The biochemistry, biology, and regulation of the ERKs have been reviewed exhaustively elsewhere (10, 11, 195).

In recent years, it has become clear that, as with yeast, multiple parallel mammalian MAPK pathways exist and that most of these, in conjunction with the nuclear yeast, multiple parallel mammalian MAPK pathways exist and that most of these, in conjunction with the nuclear factor-κB (NF-κB) pathway, are pivotal to stress and inflammatory responses rather than to mitogen responses. As mammalian stress-activated signaling pathways are elucidated, it is becoming evident that these pathways will be important targets for novel anti-inflammatory drugs.

B. The Stress-Activated Protein Kinases/c-Jun NH2-Terminal Kinases, a Family of MAPKs Activated by Environmental Stresses and Inflammatory Cytokines of the TNF Superfamily

Indications that mammalian cells possessed several MAPK pathways came shortly after the identification of the ERKs. The protein synthesis inhibitor cycloheximide, when administrated to rats, can elicit the in vivo activation of ribosomal S6 phosphorylation, and, in fact, this strategy was used to activate p70 S6 kinase in vivo before purification (10). It was originally suggested that p70 S6 kinase, like MAPK-activated protein kinase-1 (MAPKAP-K1)/ribosomal S6 kinase (Rsk), a known ERK substrate with which p70 S6 kinases shares some superficial enzymological similarities (85, 254, 292), would be activated by ERKs. However, the ERKs were unable to activate p70 S6 kinase in vitro, suggesting that cycloheximide activated novel kinases including proline-directed protein kinases (164). This idea was supported by the demonstration that injection of cycloheximide into rats activated a novel Ser/Thr kinase activity that could phosphorylate microtubule-associated protein-2 (MAP2). Purification of this kinase revealed a 54-kDa polypeptide, initially named p54-MAP2 kinase, or p54 (164, 165). p54 could be inactivated with Tyr or Ser/Thr phosphatases, indicating that, like the ERKs, the p54 kinase required concomitant Tyr and Ser/Thr phosphorylation for activity (167, 165); moreover, studies with synthetic peptide substrates showed that p54 was proline directed, but phosphorylated Ser/Thr-Pro motifs with a specificity distinct for that of the p42/p44 MAPKs (220).

The specificity of p54 toward native protein substrates clearly differed from that of the ERKs. In particular, p54 was unable to activate MAPKAP-K1/Rsk in vitro under conditions wherein ERK-mediated activation of MAPKAP-K1/Rsk was observed (164). Of note, p54 was also unable to activate p70 S6 kinase in vitro, although both p54 and the ERKs could phosphorylate p70 S6 kinase in an autoinhibitory segment. p70 S6 kinase activation was subsequently shown to require additional phosphatidylinositol (PI) 3-kinase-dependent steps (10). Most importantly, p54 was able to phosphorylate the c-Jun transcription factor at two sites (Ser-63 and Ser-73) implicated in regulation of c-Jun and AP-1 trans-activation function (244), at a substantially higher rate than was observed for the p42/p44 MAPKs.

The SAPKs were cloned independently by two groups: Kyriakis et al. (166) used the amino acid sequence of tryptic peptides derived from purified p54 to design specific PCR primers, whereas Dérijard et al. (68) used a pure PCR strategy employing degenerate primers derived from regions conserved among known MAPKs. The generation of specific antibodies enabled an analysis of the regulation of endogenous p54 by extracellular stimuli. Assay of p54 kinase activity immunoprecipitated from extracts of cells subjected to various treatments revealed that, in contrast to the p42/p44 MAPKs, p54 was not strongly activated in most cells by mitogens such as insulin, EGF, PDGF, or FGF. In contrast, p54 was vigorously activated by a variety of noxious treatments such as heat shock, ionizing radiation, oxidant stress, DNA damaging chemicals (topoisomerase inhibitors and alkylating agents), reperfusion injury, mechanical shear stress, and, of course, protein synthesis inhibitors (cyclheximide and anisomycin) (68, 165, 166, 237).

In rationalizing the significance of p54 activation by environmental stresses, the potent activation of p54 by tunicamycin was conceptually important; tunicamycin inhibits N-linked protein glycosylation and leads to the accumulation of misfolded proteins exclusively within the lumen of the endoplasmic reticulum (ER). The ability of an ER-localized perturbation to activate the cytosolic p54 kinase was one of the first clear-cut examples of stress-activated signal transduction through a protein kinase (166). This formulation suggested that stress-activated cytokines were likely regulators of p54 activity, a supposition rapidly borne out by the demonstration that p54 is strongly activated by all the inflammatory cytokines of the
TNF family [TNF, interleukin (IL)-1, CD40 ligand, CD27 ligand, Fas ligand, receptor activator of NF-κB, RANK ligand, etc.] as well as by vasoactive peptides (endothelin and ANG II) (4, 16, 19, 68, 166, 178, 275, 369). p54 has since been renamed, and the nomenclature of this family of kinases is somewhat confusing (Table 1). Two systems are generally accepted: stress-activated protein kinase (SAPK) in reference to the regulation of these kinases by environmental stress and inflammation and c-Jun NH₂-terminal kinase (JNK) in reference to the phosphorylation by these kinases of the c-Jun NH₂-terminal trans-activation domain.

The SAPKs are encoded by at least three genes: SAPKα/JNK2, SAPKβ/JNK3, and SAPKγ/JNK1 (111, 143, 166) (Table 1). As with all MAPKs, each SAPK isoform contains a characteristic Thr-X-Tyr phosphoacceptor loop in subdomain VIII of the protein kinase catalytic domain. Whereas the ERK sequence is Thr-185-Glu-Tyr-187 (ERK2) or Thr-203-Glu-Tyr-205 (ERK1), that of the SAPKs is Thr-183-Pro-Tyr-185. The expression of each SAPK gene (ERK2) or Thr-203-Glu-Tyr-205 (ERK1), that of the SAPKs contains a characteristic Thr-X-Tyr phosphoacceptor loop in subdomain VIII of the protein kinase catalytic domain. Whereas the ERK sequence is Thr-185-Glu-Tyr-187 (ERK2) or Thr-203-Glu-Tyr-205 (ERK1), that of the SAPKs is Thr-183-Pro-Tyr-185. The expression of each SAPK gene is further diversified by differential hnRNA splicing. Splicing within the catalytic domain at a region spanning subdomains IX and X results in type 1 and type 2 SAPKs (β and α JNKS, respectively, Table 1), whereas splicing at the extreme COOH terminus yields 54-kDa (p54) and 46-kDa (p46) polypeptides (type 2 and type 1 JNKS, respectively, Table 1); thus at least 12 polypeptides have been identified. The significance of the COOH-terminal isoforms is not clear, but the type 1 and type 2 kinases differ in their substrate binding affinities (63, 111, 143, 166).

### TABLE 1. MAPK nomenclature

<table>
<thead>
<tr>
<th>Name</th>
<th>Alternate Names</th>
<th>Substrates</th>
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<tr>
<td>ERK1</td>
<td>p44-MAPK</td>
<td>MAPKAP-K1, MNKS, MSKS, Elk1</td>
</tr>
<tr>
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<td>p42-MAPK</td>
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</table>

MAPK, mitogen-activated protein kinase. Commonly accepted names found in the primary literature are included. ERK pathway nomenclature is also shown but is not discussed in text (195). Not all of the names listed are included in the text.

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C. The p38 MAPKs, a Second Stress-Activated MAPK Group

The p38 MAPKs are a second mammalian stress-activated MAPK family. Originally described as a 38-kDa polypeptide that underwent Tyr phosphorylation in response to endotoxin treatment and osmotic shock (117), p38 (the α isoform) was purified by anti-phosphotyrosine immunoaffinity chromatography; cDNA cloning revealed that p38 was the mammalian MAPK homolog most closely related to HOG1, the osmosensing MAPK of S. cerevisiae. Most notably, the p38s, like Hog1p, contain the phosphoacceptor sequence Thr-Gly-Tyr (117, 120). Independently and contemporaneously, two groups identified p38α as a kinase activated by stress and IL-1 that could phosphorylate and activate MAPK-activated protein kinase-activated protein kinase-1 (MAPKAP kinase-2, see sect. iE1), a novel Ser/Thr kinase implicated in the phosphorylation and activation of the small 27-kDa heat shock protein HSP27 (95, 259).

Of potential clinical importance, p38α was also purified and cloned as the polypeptide receptor for a class of experimental pyridinyl-imidazole anti-inflammatory drugs, the cytokine-suppressive anti-inflammatory drugs (CSAIDs), the most extensively characterized of which is the compound SB203580 (174). CSAIDs were originally identified in a screen for compounds that could inhibit the transcriptional induction of TNF and IL-1 during endotoxin shock (174). The basis for the efficacy of these compounds as anti-inflammatory agents was their ability to bind and directly inhibit a subset of the p38s, thereby blocking p38-mediated activation of AP-1, a trans-acting factor required for TNF and IL-1 induction (174). Finally, a shorter hnRNA splicing isoform of p38 was isolated as a kinase that could interact with the Myc binding partner Max (362) (see sect. uE2). With the identification of additional p38 isoforms, four p38 genes are now known (Table 1): the original isoform, here referred to as p38α [also called CSAIDs binding protein (CSBP) and, somewhat confusingly, SAPK2α], p38β (also called SAPK2b, and p38–2), p38γ (also called SAPK3 and ERK6), and p38δ (also called SAPK4) (95, 106, 117, 139, 140, 173, 174, 207, 259, 288, 327, 363).

In vitro assays demonstrated that only p38α and p38β are inhibited by CSAIDs; p38γ and p38δ are completely unaffected by these drugs in vitro or in transduced cells (106). The basis for this inhibition was revealed in the crystal structure of p38α complexed with the SB203580. Thr-106 in the hinge of the p38α ATP binding pocket interacts with a fluorine atom in the SB203580 structure.
This orients the drug to interact with His-107 and Leu-108 of the ATP binding pocket (86, 110). Substitution of Thr-106, alone or in combination with His-107 or Leu-108, with the corresponding, more bulky residues from p38α or p38β (Met, and Pro or Phe, respectively, in both cases) abolishes SB203580 binding. Conversely, if the amino acid of p38γ, p38δ, or even SAPKα which corresponds to p38α Thr106 is replaced with Thr, the resulting mutants display at least partial sensitivity to SB203580 (86, 110).

Like the SAPKs, the p38s are strongly activated in vivo by environmental stresses and inflammatory cytokines and are inconsistently activated by insulin and growth factors. In almost all instances, the same stimuli that recruit the SAPKs also recruit the p38s (165). One exception is ischemia-reperfusion. SAPKs are not activated during ischemia, but rather during reperfusion, whereas the p38s are activated during ischemia and remain active during reperfusion (22, 165, 237). The basis for this difference is unknown.

D. ERK5/Big MAP Kinase-1 (BMK1), a Third Class of Stress-Activated MAPK

The novel MEK MEK5 was cloned by degenerate PCR as part of an effort to identify new MAPK pathways and regulators (83, 367). ERK5, a MEK5 substrate, was cloned as part of a two-hybrid screen that employed MEK5 as bait (367). ERK5 is a ∼90-kDa MAPK of which only one mammalian homolog is known. ERK5 has the sequence Thr-Glu-Tyr in its phosphoacceptor loop. The NH2-terminal kinase domain of ERK5 is followed by an extensive COOH-terminal tail of unknown function that contains several consensus proline-rich motifs indicative of binding sites for proteins with SH3 domains (367).

The stimuli that recruit ERK5 have not been comprehensively characterized; however, ERK5 can be substantially activated by environmental stresses such as oxidative stress (peroxide) and osmotic shock (sorbitol), but not by vasoactive peptides or inflammatory cytokines (TNF) (1). ERK5 may also lie downstream of receptor Tyr kinases. Although mitogen activation of ERK5 was not initially observed (1), EGF activation of ERK5 has been subsequently documented (38).

E. SAPK, p38, and ERK5 Substrates

As with the ERKs, the SAPKs, p38s, and ERK5 phosphorylate both transcription factors and other protein kinases. Some of the protein kinase substrates (MAPKAP-K2 and -K3 and PRAK) are selectively recruited by stress-activated MAPKs. Others (MNKs and MSKs), however, are activated by both stress- and mitogen-regulated MAPKs and, like the AP-1 transcription factor, integrate both stress and mitogenic signaling pathways. The substrates of stress-activated MAPKs highlight the importance of these MAPKs to the inflammatory response.

1. Protein kinases

A) MITOGEN-ACTIVATED PROTEIN KINASE-ACTIVATED PROTEIN KINASES (MAPKAP-KS)-2 AND -3. MAPKAP kinase-2 (MAPKAP-K2) and the structurally related MAPKAP-K3 (also called three pathway regulated kinase or 3PK) are a small family of Ser/Thr kinases that consist of an NH2-terminal regulatory domain and a COOH-terminal kinase domain (132, 169, 203, 283, 289, 290). They are unrelated to the MAPKAP-K1s/Rsks, targets of the ERKs (11, 85). Along with p38-regulated and activated kinase (PRAK, see below), MAPKAP-K2 and MAPKAP-K3 phosphorylate the small heat shock protein HSP27 (132, 169, 203, 283, 289, 290). Nonphosphorylated HSP27 normally exists in high-molecular-weight multimers that serve as molecular chaperones. Phosphorylation of HSP27 by MAPKAP kinase-2/3 at Ser-15, Ser-78, Ser-82, and Ser-90 coincides with the dissociation of HSP27 into monomers and dimers, and with the redistribution of HSP27 to the actin cytoskeleton (132). In peroxide-treated human umbilical vein endothelial cells, this redistribution of HSP27 may contribute to triggering the reorganization of F-actin into stress fibers, thereby affecting cell motility (132, 169). MAPKAP-K2/3-catalyzed phosphorylation of HSP27 at Ser-90 appears necessary for this process, and mutation of Ser-90 to Ala prevents stimulus-induced changes in HSP27 oligomerization (169).

Early studies had indicated that purified ERK1 could phosphorylate and activate MAPKAP-K2 in vitro (289). However, ERKs likely do not represent the physiological MAPKAP-K2 kinases. MAPKAP-K2 is activated not by insulin or mitogens, conditions wherein ERKs are strongly activated, but by stresses and inflammatory cytokines, conditions wherein ERKs are not appreciably activated (95, 259). MAPKAP-K2 is phosphorylated and activated by p38α and p38β (but not by p38γ or p38δ) (60, 95, 259) (Fig. 2). Activation of MAPKAP-K2 is a multistep process wherein phosphorylation of Thr-222, catalyzed by either p38α or p38β, gates subsequent phosphorylation of Thr-222 and Ser-272 in the kinase activation loop, a reaction also catalyzed by either p38α or p38β. Together, these phosphorylations, accompanied by an additional autophosphorylation at Thr-334, result in activation of MAPKAP-K2 (15). Consistent with regulation by p38α and p38β, MAPKAP-K2 activation and HSP27 phosphorylation are inhibited by CSAIDs (15, 132, 169, 203).

MAPKAP-K3 can also phosphorylate HSP27 (169). Although it has been shown that MAPKAP-K3 can be activated in vivo, in overexpression experiments, by the ERK, SAPK, and p38 pathways (189), endogenous MAPKAP-K3 is only significantly activated by stresses and inflammatory cytokines, and in a manner that can
be completely inhibited with CSAIDs, suggesting that MAPKAP-K3 is, in fact, stress and not mitogen activated, and that p38α and/or p38β are the major MAPKAP-K3 kinases in vivo (169).

b) p38-regulated/activated kinase. p38-regulated/activated kinase (PRAK) is a ~50-kDa Ser/Thr kinase with a similar overall structure to MAPKAP-K2, -K3, and the MAPK-interacting kinases (MNKs, see below). Accordingly, PRAK consists of an NH2-terminal regulatory domain and a COOH-terminal kinase domain. Like MAPKAP-K2 and -K3, PRAK is activated selectively in response to stress and inflammatory cytokines and is not detectably activated by mitogens. PRAK can be activated in vivo and in vitro by p38α and p38β, and consistent with this, PRAK activation can be blocked by CSAIDs (224). Phosphorylation of PRAK catalyzed by p38α is at Thr-182 in the activation loop (224). Once activated, PRAK can phosphorylate HSP27 at the physiologically relevant sites, and in-gel kinase assays indicate that PRAK is an important stress-activated HSP27 kinase (224) (Fig. 2).

c) MNKs. Cellular mRNAs contain a 5′-cap structure, the N7-methylguanosine cap. The N7-methylguanosine-binding protein eIF-4E recruits mRNAs onto a scaffold protein eIF-4G, which also binds the RNA helicase eIF-4A. The latter, in collaboration with the RNA binding protein eIF-4B, unwind secondary structure in the mRNA 5′-untranslated segment, thereby facilitating the scanning of the mRNA by the 40S ribosomal complex to the ATG translational initiation site. The complex of eIF-4A, B, G, and E is known as eIF-4F (285).

The eIF-4E-eIF-4G interaction is negatively regulated by the translational repressor protein 4E-binding protein-1 (4E-BP1 also called phosphorylated heat- and acid-stable protein regulated by insulin, PHAS-I). 4E-BP1 is multiply phosphorylated in response to insulin and mitogens resulting in the dissociation of 4E-BP1 from eIF-4E, and the release of eIF-4E for incorporation into the eIF-4F complex. In vivo, the phosphorylation of eIF-4E is strongly inhibited by rapamycin and wortmannin, consistent with the fact that dissociation of 4E-BP1 is regulated by the mammalian target of rapamycin (mTOR) (11, 285) as well as by protein kinases downstream of PI 3-kinase (285).

In addition, eIF-4E itself also undergoes a regulatory phosphorylation at Ser-209 in response to both insulin/mitogen and environmental stress. This phosphorylation increases the affinity of eIF-4E for the 5′-cap by about threefold; crystallographic data also indicate that phosphorylation of eIF-4E favors the binding of 4E to the 5′-cap (285).

MNK-1 and -2 are two closely related kinases that are probably the physiologically relevant eIF-4E Ser-209 kinases. As the name implies, MNKs associate in vivo with MAPKs and are in vitro and in vivo MAPK substrates. MNKs are phosphorylated and activated both by ERKs 1/2 (in response to insulin and mitogens) and by the p38s (in response to stress) (98, 331, 332). The regulation of the MNKs by both the ERKs and p38s indicates that, as with AP-1 (see below), MNKs are a site of integration of stress and mitogenic signaling pathways (Fig. 2).
activation of MSK1 in HeLa cells. After 15 min of TNF stimulation, however, SB203580 inhibition is only partial, and MSK1 can now also be partially inhibited with the ERK pathway inhibitor PD98059 (66) (Fig. 2).

cAMP response element binding protein (CREB) is a bZIP transcription factor that binds and trans-activates genes containing the cAMP response element (CRE—consensus sequence: TGACGTCA). CREB trans-activating activity can be activated by a number of different stimuli including mitogens, stresses, and, of course, agonists that elevate cAMP. Activation of CREB trans-activating activity correlates with phosphorylation at Ser-133. Protein kinase A (PKA) likely represents the major CREB kinase recruited by cAMP (113). In addition, several protein kinases including MAPKAP-K1s/Rsks (343) and MAPKAP-K2 (300) have been shown to possess mitogen- or stress-activated CREB Ser-133 kinase activity. MSK1 is also a potent CREB kinase in vitro, and a substantial amount of evidence indicates that, in fact, MSK1, and not MAPKAP-K1/Rsk or MAPKAP-K2/3, is the physiologically relevant stress- and mitogen-activated CREB kinase.(66).

First, the \(K_{cat}\) for MSK1-catalyzed phosphorylation of CREB is nearly 100-fold greater than that for MAPKAP-K1/Rsk-catalyzed CREB phosphorylation. Moreover, the MSK1 polypeptide has a bipartite nuclear localization signal and is localized exclusively in the nucleus, where CREB resides. MAPKAP-K1s/Rsks are largely cytosolic, although they can translocate to the nucleus in response to extracellular stimuli (40). Finally, the pattern of pharmacological inhibition of stress- and mitogen-activated CREB phosphorylation in vivo tightly corresponds to the pattern of pharmacological inhibition of MSK1 activation. Thus activation of CREB by stress and mitogens is blocked by the broad specificity kinase inhibitor Ro318220. MSK1 is strongly inhibited in vitro by Ro318220, whereas MAPKAP-K2 and MAPKAP-K3 are not. Finally, CREB activation in vivo by TNF can be blocked with SB203580 and the ERK inhibitor PD98059 with kinetics that parallel those described above for inhibition of MSK1 (66).

A recent study suggests that MSKs may be involved in the phosphorylation of components involved in chromatin remodeling. Both mitogens and stresses, as they stimulate gene expression, must prompt the loosening of chromatin structure from around target genes. This enables the transcriptional machinery to gain access to genes that are to be expressed (35, 267). This alteration in chromatin structure involves both the acetylation and phosphorylation of histones. Many transcription factors that are phosphorylated in a stimulus-dependent manner can recruit transcriptional coactivators that are histone acetyl transferases. In addition, protein kinase cascades phosphorylate histones themselves (35, 267). The nucleosomal proteins histone H3 and high mobility group-14 (HMG-14) are prime targets of phosphorylation (35, 267, 308). Agonist-stimulated phosphorylation of histone H3 occurs at Ser-10, while HMG-14 is phosphorylated at Ser-6. Recent genetic evidence indicates that mitogenic histone H3 phosphorylation is mediated at least in part by MAPKAP-K1b/Rsk2 inasmuch as cells derived from patients with Coffin-Lowry syndrome, a loss of function mutation in the \(\text{mapkap-k1b/rsk2}\) gene, display a substantial deficit in mitogen-stimulated histone H3 phosphorylation (267).

However, it is unclear if MAPKAP-K1b/Rsk2 is the only histone H3 kinase in vivo. Thus both mitogens and stresses can stimulate H3 and HMG-14 phosphorylation in vivo (308), and MAPKAP-K1s/Rsks are not strongly activated by stresses (10, 164). Moreover, MAPKAP-K1s/Rsks are poor HMG-14 kinases in vitro and are not inhibited by the inhibitor H89 which blocks HMG-14 kinase activity in vivo and in vitro. In contrast, MSK1 is strongly inhibited by H89 in parallel with in vivo inhibition of histone H3 and HMG-14 phosphorylation. In addition, in vitro, MSK1 can phosphorylate histone H3 at Ser-10, at a rate comparable to or better than that phosphorylated by MAPKAP-K1s/Rsks in vitro (308).

2. Transcription factors

A) CREB HOMOLOGOUS PROTEIN/GROWTH ARREST AND DNA DAMAGE-155. CREB homologous protein (CHOP)/growth arrest and DNA damage-155 (GADD153) is a bZIP transcription factor of the CREB family (113). CHOP/GADD153 is transcriptionally induced in response to genotoxic and inflammatory stresses. These treatments can also activate the transcriptional regulatory functions of CHOP/GADD153 through stimulus-induced phosphorylation of Ser-78 and Ser-81. CHOP/GADD153 is a transcriptional repressor of certain cAMP-regulated genes and a transcriptional activator of some stress-induced genes. Activation by genotoxins of CHOP/GADD153 mediates in part cell cycle arrest at G1/S, an important consequence of DNA damage inasmuch as it allows for DNA repair before DNA replication, key to preserving genomic integrity. p38\(\alpha\) is a likely stress-activated regulator of CHOP/GADD153 function, given that p38\(\alpha\), and not SAPK or ERK, can phosphorylate CHOP/GADD153 at Ser-78 and Ser-81 in vivo and in vitro (325) (Fig. 3).

B) NUCLEAR FACTOR OF ACTIVATED T CELLS. Transcription factors of the nuclear factor of activated T cells (NFAT) family are distantly related to Rel/NF-kB (250). In resting cells, NFATs are retained in the cytosol as a consequence of phosphorylation [catalyzed by casein kinase-I\(\alpha\) and, possibly, glycogen synthase kinase (GSK)-3] at five or six sites (within NFAT4, this comprises a region spanning amino acids 204–215). NFAT phosphorylation affects conformation so as to mask the nuclear localization signal. Agonist-induced Ca\(^{2+}\) entry recruits the Ca\(^{2+}\)-dependent phosphatase calcineurin (phosphatase 2B), which dephosphorylates NFATs, thereby exposing NFAT's nuclear
Localization signal and triggering NFAT nuclear translocation. Dephosphorylation of NFATs also enhances DNA binding affinity (250). NFATs bind and trans-activate genes with an NFAT cis-acting element (consensus sequence: T/GGAAAAT). NFAT sites are often located close to AP-1 sites in many promoters, allowing for the cooperative binding and synergistic trans-activation of numerous genes (IL-2, IL-4, IL-5, and CD40L are examples) (250). Calcineurin is a major target of the immunosuppressants FK506 and cyclosporin A, and accordingly, inhibition of NFAT activity is an important consequence of FK506 and cyclosporin A action (250, 270).

Serum factors can substantially inhibit Ca\(^{2+}\)-mediated nuclear translocation of NFATs. Insofar as casein kinases and GSK3 are not serum stimulated, the serum-dependent inhibition of Ca\(^{2+}\)-mediated NFAT translocation suggested that serum-responsive kinase cascades might contribute to NFAT inhibition (47, 250). Davis and colleagues (47) showed that the SAPKs could phosphorylate the NFAT family member NFAT4 (also called NFATc3) at Ser-163 and Ser-165. This phosphorylation correlates with an inhibition of stimulus-induced NFAT4 nuclear translocation, and on the basis of this finding, it has been proposed therefore that the SAPK pathway antagonizes NFAT4 action (47) (Fig. 3). However, the significance of these results is somewhat unclear. Zhu et al. (368) showed that NFAT4 mutants in which the putative SAPK phosphoacceptor sites (Ser-163 and Ser-165) have been changed to Ala still show serum-stimulated inhibition of nuclear translocation in many instances. These investigators also observed that the SAPK-specific MAP3K MEK-kinase-1 (see sect. 11G2) could indirectly, and independently of SAPK, block NFAT4 dephosphorylation and activation. MEKK1 inhibition of NFAT translocation occurs whether or not the SAPK phosphorylation sites have been mutated to alanine, and the effect of MEKK1 is not reversed by dominant inhibitors of the SAPK pathway. Apparently, MEKK1 fosters inhibition of NFAT nuclear translocation by stabilizing the association between NFAT4 and the inhibitory NFAT kinase casein kinase-I\(\alpha\) (368).

Davis and colleagues (48) have also shown that SAPKs can phosphorylate NFATc1 (also called NFAT2). In this instance, phosphorylation is stimulated by phorbol 12-myristate 13-acetate (PMA) and ionomycin and occurs at Ser-117 and -172. Phosphorylation inhibits or delays the accumulation of NFATc1 in the nucleus by blocking the binding of calcineurin, an essential step in NFATc1 activation (48). NFATc1 is crucial to the differentiation of TH cells to the TH2 effector phenotype (see sect. V1F1). Interestingly, disruption of SAPK\(\gamma\) or both SAPK\(\gamma\) and -\(\alpha\) leads to the preferential accumulation of TH2 cells, consistent with the notion that SAPK-mediated inhibition of TH2 differentiation is mediated through NFATc1 (48, 76, 77) (see sect. V1F1).

c) Max. Max is a 12-kDa helix-loop-helix (HLH) polypeptide that interacts with the transcription factor c-Myc enabling c-Myc to trans-activate at least a subset of its target genes. c-Myc is a key regulator of cell proliferation, differentiation, and apoptosis. The biological functions of c-Myc are thought to depend in part on the polypeptide binding partners with which c-Myc interacts and the regulation of these binding partners (20). A COOH-terminally truncated hnRNA splicing isoform of p38\(\alpha\), referred to as Max-interacting protein-2 (Mxi2), was isolated in a yeast two-hybrid screen for Max interactors.

![Diagram of MAPK signaling pathways](http://physrev.physiology.org/article/download/10.220.33.5/815/MAMMALIAN_MAPK_SIGNAL_TRANSDUCTION_PATHWAYS/3.png)
Max and either Mxi2 or p38α can form a tight complex in vivo and in vitro, an interaction thought to be mediated by the HLH domain of Max and a similar loop on Mxi2 and p38α. Max is a good substrate for both Mxi2 and p38α (363). Interestingly, there are no consensus proline-directed sites on Max, suggesting that the absolute proline requirement for MAPKs may be less pronounced for p38. The functional significance of p38-catalyzed Max phosphorylation is unclear (363) (Fig. 3).

**d) Activator Protein-1.** The SAPKs and p38α are the dominant stress-activated Ser/Thr kinases responsible for the recruitment of the activator protein-1 (AP-1) transcription factor (147, 165). ERK5 also plays a role in stress-activated AP-1 regulation (150). AP-1 is a heterodimer comprised of bZIP transcription factors, typically c-Jun and JunD, along with members of the fos (usually c-Fos) and ATF (usually ATF2) families. All bZIP transcription factors contain leucine zippers that enable homo- and heterodimerization, and AP-1 components are organized into Jun-Jun, Jun-Fos, or Jun-ATF dimers (147) (Fig. 4).

The presence of Jun family members enables AP-1 to bind to cis-acting elements containing the 12-O-tetradecanoylphorbol 13-acetate (TPA) response element (TR; consensus sequence: TGA^C/C^G TCA). ATFs, including ATF2, are members of the CREB subfamily of bZIP transcription factors. AP-1 heterodimers containing ATF transcription factors can bind both to the TRE and to the CRE (113, 147). AP-1 is an important trans-activator of a number of stress responsive genes including the genes for IL-1 and -2, CD40, CD30, TNF, and c-Jun itself. In addition, AP-1 participates in the transcriptional induction of proteases and cell adhesion proteins (e.g., E-selectin) important to inflammation (147, 252).

Activation of AP-1 involves both the direct phosphorylation/dephosphorylation of AP-1 components as well as the phosphorylation and activation of transcription factors that induce elevated expression of c-jun or c-fos. Both events can be activated independently by several signaling pathways (Fig. 4). Thus c-Jun is phosphorylated in resting cells at a region immediately upstream of the COOH-terminal, the DNA binding domain (Thr-231, Thr-239, Ser-243, Ser-249). This phosphorylation inhibits DNA binding and can be catalyzed in vivo by either glycogen synthase kinase-3 (GSK3) or casein kinase II (CKII). Upon stimulation of cells with AP-1 activators, the c-Jun COOH-terminal phosphates are removed under conditions wherein GSK3 is inactivated (24, 147). GSK3 is inhibited upon mitogen stimulation by a mechanism dependent on PI 3-kinase (58, 79).

Phosphorylation of c-Jun or ATF2 within their NH₂-terminal trans-activation domains correlates well with enhanced trans-activating activity (68, 112, 160, 244). The SAPKs can phosphorylate the c-Jun trans-activating domain at Ser-63 and Ser-73 (68, 166, 244). These residues are phosphorylated in vivo under conditions wherein the SAPKs are activated. Immunodepletion of SAPK from cell extracts removes all stress- and TNF-activated c-Jun kinase (166, 237). Thus SAPKs are the dominant kinases responsible for stress- and TNF-activated c-Jun phosphorylation (Fig. 2). JunD is also phosphorylated at Ser-90 and Ser-100 by SAPKs, albeit less effectively than is c-Jun. Ser-90 and Ser-100 of JunD lie within a region of the JunD trans-activation domain similar to the phosphoacceptor domain of c-Jun (144).

The SAPKs and p38α can both phosphorylate ATF2 at Thr-69 and Ser-71 in the trans-activation domain. Again, these residues are phosphorylated in vivo under conditions in which the SAPKs and p38α are activated. Phosphorylation of ATF2 at Thr-69 and Ser-71 correlates with activation of ATF2 trans-activating activity (112) (Fig. 3). Whether the SAPKs or p38α represent the dominant ATF2 kinases depends on the cell type and stimulus used. During reperfusion of ischemic kidney, for example, the SAPKs are the only detectable ATF2 kinases (219), whereas in KB keratinocytes treated with IL-1, the p38α are the major ATF2 kinases (60).

The SAPKs, p38α, and ERK5 (primarily in response to stress) and the ERKs (primarily in response to mitogens) also contribute to AP-1 activation by stimulating the transcription of genes encoding AP-1 components (147, 150) (Fig. 3). Induction of c-fos expression is one of the earliest transcriptional events known to occur. The fos promoter includes a cis-acting element, the serum response element (SRE), that mediates the recruitment of a heterodimeric transcription factor containing two polypeptides, the serum response factor (SRF) and a member of the ternary complex factor (TCF) family (318) (Fig. 3).

The TCFs include Elk-1 and Sap-1a (318). The SAPKs, and ERKs, but not p38, can phosphorylate two critical residues in the Elk1 COOH terminus (Ser-383, Ser-389), while the p38α can efficiently phosphorylate the corresponding residues (Ser-381 and Ser-387) on Sap1a (104, 138, 194, 337, 351). This phosphorylation enhances the binding of TCFs to the SRF and thereby triggers trans-activation at the SRE. By these processes, MAPKs activated by both stress and mitogens can convergently contribute to c-fos induction (Fig. 3).

The p38α and ERK5 can also phosphorylate the transcription factors myocyte enhancer factor-2 MEF2 subgroup of the MCM1-agamous and deficiens-SRF (MADS) box transcription factor family (116, 150, 365). MEF2s (MEF2A-D) were originally identified as transcription factors that bound to AT-rich sequences (consensus: CTAAAATAA) and trans-activated key genes involved in myoblast differentiation; however, some MEF2s, notably MEF2C, are widely expressed and may regulate numerous other transcriptional regulatory events (94). Only MEF2A and -C are MAPK substrates. MEF2B and -D are not MAPK substrates; however,
MEF2B and -D may act in conjunction with phosphor-
ylated MEF2A/C. Thus phosphorylation enhances the
trans-activating activity of MEF2A and -B or A and D
heterodimers (365). MEF2A is phosphorylated at Thr-
312 and Thr-319 by p38α. Phosphorylation by other
MAPKs has not been observed (365). MEF2C phosphor-
ylation by MAPKs is apparently more complex. The
p38α and p38α MAPKs phosphorylate different sets of sites on
the MEF2C polypeptide. Thus p38α phosphorylate Thr-
293 and Thr-300, whereas ERK5 phosphorylates Ser-387
(116, 150). All three sites are phosphorylated in re-
sponse to serum or stress; however, Thr-293/Thr-300
phosphorylation is sufficient for p38α activation of
MEF2C while Ser-387 phosphorylation is sufficient for
ERK5 activation of MEF2C (116, 150). A

AP-1 activation. Indeed, MEF2A or -C, once activated by p38s, can trans-activate the c-Jun promoter (116, 150, 365) (Fig. 3).

3. The substrate specificity of the SAPKs and p38s is conferred by specific MAPK docking sites on substrate proteins: the MAPK docking sites interact with specific substrate binding motifs on MAPK polypeptides

The phosphorylation of c-Jun by the SAPKs highlights an important point about the mechanism of substrate recognition by members of the MAPK family. All MAPKs are "proline-directed," phosphorylating Ser/Thr residues only if followed immediately by proline (see sect. 1 and Fig. 4). However, the specificity of MAPKs for their physiological substrates is dictated in large part by the presence of binding sites, often substantially distal from the phosphorylation sites, that are specific for distinct MAPK subgroups. These MAPK binding sites allow for the selective interaction between MAPKs and their true in vivo substrates.

Two general classes of MAPK binding site have been described for MAPK substrates. The first of these to be identified was a hydrophobic cluster of residues present in many transcription factor substrates of MAPKs. Thus the SAPKs, but not the ERKs or p38s, bind c-Jun quite strongly. The SAPK binding site on c-Jun lies between residues 32 and 52, well away in the primary sequence from Ser-63 and Ser-73, the sites of phosphorylation (63, 143, 144, 147). The SAPK docking site overlaps with the so-called δ-domain (amino acids 30–57), a hydrophobic region initially implicated in the regulation of c-Jun oncogenicity due to its deletion in oncogenic v-Jun (accordingly, v-Jun does not bind SAPK and is not a SAPK substrate) (63, 143, 144, 147) (Fig. 4).

The binding of c-Jun to SAPK isoforms has been mapped to a β-strand-like region spanning subdomains IX and X. Interestingly, a portion of this region undergoes differential hnRNA splicing in type I and II SAPKs (63, 111, 143, 166) (Table 1 and sect. II). This β-strand region also differs substantially among the three SAPK genes, perhaps accounting for differential SAPK substrate selectivity. Thus SAPKα interacts most strongly with c-Jun (63, 111, 143).

The presence of the SAPK binding site, in conjunction with the ability of c-Jun to heterodimerize with other members of the Jun family, enables the SAPKs to phosphorylate other AP-1 constituents in vivo that, as monomers or homodimers, are poor SAPK substrates (144, 147). Thus JunD possesses domains similar to the phosphoacceptor and SAPK binding domains of c-Jun. In spite of this, JunD binds SAPK poorly (144) (Fig. 4). Accordingly, purified JunD is not ordinarily a SAPK substrate in vitro; however, when heterodimerized with c-Jun, JunD can undergo efficient SAPK-catalyzed phosphorylation in vitro, and activation in transfection experiments in vivo (144) (Fig. 4). JunB also possesses a conserved region homologous to the SAPK binding pocket of c-Jun, and in contrast to JunD, JunB binds SAPK well. However, JunB does not possess the proline-directed phosphoacceptor sites that are required for SAPK phosphorylation (144, 147) (Fig. 4A). Thus JunB is not a SAPK substrate in vivo or in vitro (144) (Fig. 4B).

ATF2 also contains a hydrophobic pocket (amino acids 20–60) similar to the c-Jun δ-domain that binds SAPKs and p38s. As with the c-Jun δ-domain, the ATF2 MAPK binding site lies NH2 terminal to the phosphoacceptor sites (Thr-69 and Thr-71) (112). Likewise, Elk-1 has a MAPK docking site, the D-domain (amino acids 312–334) that lies NH2 terminal to the phosphoacceptor sites (Ser-383, Ser-389) (337, 351). Interestingly, the Elk-1 D-domain is essential for ERK and SAPK binding and phosphorylation; however, p38 binding and phosphorylation, the physiological significance of which has not been unambiguously established (138, 351), appears not to require this domain (351). The role of the SAPK β-strand in determining the differential binding of different SAPK isoforms to ATF2 or Elk1 is unknown.

A second class of MAPK binding domain generally consists of a small stretch of basic residues (Fig. 4C). This domain is prevalent among protein kinase substrates of MAPKs, including MAPKAP-K1s/Rsks (ERK substrates), MNks, MAPKAP-K2/3, and PRAK and binds to the recently identified common docking (CD domain) domain, an extracatalytic region rich in acidic residues, found in all MAPKs (301). Interestingly, the basic MAPK binding motif is found not only in many MAPK substrates, but in a diverse array of MAPK regulators including MEKs (notably the SAPK-specific MEKs SAPK/ERK kinase-1, SEK1 and some MAPK-kinase-7, MKK7, isoforms, as well as the p38 MEKs MKK3 and MKK6) and MAPK phosphatases (301) (Fig. 4C). It is likely that the basic residues in these novel MAPK binding sites interact electrostatically with the cognate MAPK CD motifs. Moreover, differences among the basic MAPK docking sites and MAPK CD domains likely confer a high degree of specificity among MAPKs for their substrates and activators. This is readily apparent in the specific activation of MAPKAP-K2, under initial rate conditions, by p38 (see sect. II), and in the highly selective scaffolding function of SEK1 (see sect. III).
4. The three known SAPK genes and differential hnRNA splicing generate SAPK isoforms with different functions

As discussed in section A, the SAPKs are encoded by three genes that undergo differential hnRNA splicing to generate at least 10, and possibly up to 12, polypeptide species (111, 166). As noted in section A, there is some evidence that the hnRNA splicing in the SAPK β-strand region spanning kinase subdomains IX-X that generates the type 1 and type 2 enzymes (Table 1) may affect the affinity of different SAPK splicing isoforms for substrate. Thus SAPK-p54α2 (JNK2α1, Table 1) binds more strongly to c-Jun than does SAPK-p54α1 (JNK2β2, Table 1). Moreover, SAPKα type 2 (JNK2 type α, Table 1) isoforms bind more strongly to c-Jun than to ATF2, whereas SAPKα type 1 (JNK2 type β) isoforms bind more strongly to ATF2 than to c-Jun (63, 111, 143).

In addition to differences in substrate binding among splicing isoforms of the same SAPK gene, the different sapk gene products themselves, SAPKα, β, and γ (JNKs-2, -3 and -1, respectively, Table 1), may also display differential substrate selectivity. Thus, whereas all SAPK isoforms bind more strongly to c-Jun compared with ATF2, SAPKα/JNK2 isoforms are overall higher affinity c-Jun kinases than are either SAPKβ/JNK3 or SAPKγ/JNK1, a feature likely attributable to the high affinity for c-Jun conferred by the β-strand region in domains IX-X of the SAPK α-isofoms (Table 1) (63, 111, 143). It should be noted, however, that these differences as well as those among the splicing isoforms are modest (2- to 5-fold in vitro), and it is unclear what the in vivo biochemical significance of these differences is. These relatively subtle effects may have more profound ramifications at the cellular and organismal level. As we shall see in section V, more dramatic differences among SAPK isoforms are observed when the biological functions of these enzymes are examined.

5. The regulation of AP-1 by different classes of extracellular stimuli involves the integration of several MAPK pathways and is mediated by divergent AP-1 component transcription factor subunits

The different aspects of AP-1 regulation, activation of constituent transcription factor expression and direct phosphorylation/activation of constituent transcription factors, can be independently regulated by several pathways in response to different types of stimuli (Fig. 9). Thus mitogenic stimuli, which preferentially recruit the ERKs and inhibit GSK3, will preferentially activate AP-1 through enhancement of expression of AP-1 components (via ERK phosphorylation of Elk-1, resulting in c-fos expression, for example), and through the relief of GSK3-mediated inhibition of c-Jun DNA binding (Fig. 3).

In contrast, stresses and inflammatory cytokines such as TNF, which preferentially activate the SAPKs and p38s, can recruit AP-1 through the direct phosphorylation of AP-1 components (c-Jun by the SAPKs and ATF2 by both the SAPKs and p38s). However, stress pathways can also promote enhanced expression of AP-1 components through recruitment of Elk-1 (mediated by SAPK phosphorylation), which results in elevated c-fos expression, and through p38- and ERK5-catalyzed phosphorylation of MEF2A/C (Fig. 3). MEF2A/C can bind and trans-activate the promoter for c-jun. Stresses can also modestly recruit PKB/Akt, which can act to inhibit GSK3, thereby blocking its negative regulation of c-Jun DNA binding. Finally, the c-Jun promoter also contains an AP-1 site; thus c-jun expression can be autoregulated by any pathway that activates AP-1 (Fig. 3).

6. Blazing a trail to the nucleus: the regulation of MAPKs by nucleocytoplasmic shuttling: lessons from the ERK pathway

The ERKs, SAPKs, and p38s were first purified from cytosolic extracts and, indeed, a substantial subset of MAPK substrates are cytosolic (95, 117, 164, 174, 251, 259). Still, a great many nuclear substrates for MAPKs have been identified, and the critical question is how MAPKs localized in the cytosol could regulate nuclear proteins such as transcription factors.

Of the mammalian MAPKs, the regulation of ERK nucleocytoplasmic shuttling has been studied the most extensively, and it is likely that many of the characteristics of the regulation of ERK subcellular localization may be applicable to stress-regulated MAPKs. Early immunocytochemical examination of the subcellular localization of ERKs revealed that upon mitogen stimulation, a substantial portion of the pool of ERK immunoreactivity translocates to the nucleus. Similar stimulus-dependent translocation of the SAPKs and p38s was subsequently observed (31, 40, 247). This translocation is reversible and terminates upon cessation of stimulus (40). The potential importance of ERK nucleocytoplasmic shuttling became evident when it was observed that ERK-dependent de novo gene expression correlated with prolonged ERK activation, conditions that coincided with nuclear translocation (26, 74, 317).

Nuclear translocation is essential for ERK-dependent activation of gene expression and regulation of the cell cycle. SAPK and p38 translocation may be similarly important. Thus sequestration of ERK in the cytosol, by coexpression with a catalytically inactive form of the MAPK phosphatase (MKP) MKP3, has no effect on ERK-dependent Rsk activation or phosphorylation of an engineered cytosolic mutant of Elk-1, but strongly inhibits ERK-dependent gene transcription and S phase entry (26).

The molecular mechanism by which ERK nuclear
localization is regulated is still incompletely understood. Several novel findings suggest that inactive ERK is retained in the cytosol as part of a complex with its immediate upstream activator MEK1. Formation of this complex requires a polybasic amino acid ERK binding domain located within the NH2-terminal 32 amino acids of MEK1 (97, 301). As noted above, similar binding sites for SAPK exist on at least a subset of SAPK-specific MEKs including SAPK/ERK kinase-1 (SEK1), MAPK-kinase-7 (MKK7), as well as the p38-specific MEKs MKK3 and MKK6 (see sects. E3, F1, F2, and C4). These MAPK binding sites correspond to the basic motifs present on many MAPK protein kinase substrates (301). MEK1 itself is retained in the cytosol by a consensus nuclear export signal (amino acids 33–44). In addition, MEK cytosolic localization may be maintained by scaffold proteins, several of which have been identified for the SAPKs (see sect. C). Dissociation of the MEK-ERK complex requires MEK-catalyzed phosphorylation of ERK at the Thr-Glu-Tyr phosphoacceptor region. Once dissociation of the complex occurs, activated ERK translocates to the nucleus (97).

Whereas ERK nuclear translocation requires phosphorylation to enable dissociation from MEK1, ERK catalytic activity is not required for translocation. Both wild-type and kinase-inactive (Lys45Arg) ERK2, phosphorylated in bacteria upon coexpression with activated MEK1, could translocate to the nucleus upon microinjection into cells. In contrast, mutation of the ERK2 phosphoacceptor sites to nonphosphorylatable residues (Thr185Ala/Tyr187Phe) completely abrogated the ability of these kinases to translocate upon injection into fibroblasts (158).

Once the MEK-ERK complex dissociates, there is some evidence that a portion of the pool of ERK (primarily an active fraction that is not dimeric, see below) can freely diffuse into the nucleus. However, a substantial pool of ERK is translocated into the nucleus as part of a tightly regulated mechanism (3, 97, 158). ERK2 activation-dependent dimerization is also critical for translocation. Thus the crystal structure of activated ERK2 revealed a dimer with the interface occurring to the rear of the catalytic cleft between the two kinase lobes (28, 158). Dimerization requires four Leu residues (Leu-333, Leu-336, Leu-341, Leu-344) plus His-176 that forms a salt bridge with Glu-343 (28, 158). Mutation or deletion of the critical residues of the dimer interface has no effect on the in vitro kinase activity or MEK-catalyzed phosphorylation of the ERK2, but abolishes completely ERK2 nuclear translocation (158). Thus phosphorylation and activation of ERK2 results in dissociation from MEK1 and dimerization, both of which are necessary for translocation. It is noteworthy that both the SAPKs and p38s have similar dimerization motifs and show similar ligand-dependent translocation (31, 158, 247). Indeed, as is described in section C4, SAPKs form reversible, activation-dependent associations with the SAPK-specific MEK SEK1, complexes that may serve in part to retain inactive SAPKs in the cytosol (301, 342). The ERKs, SAPKs, and p38s do not possess consensus nuclear localization motifs, and the mechanism by which ERKs traverse the nuclear pore complex is unclear. It is conceivable that MAPKs, once liberated from elements restraining them in the cytosol, move into the nucleus by interacting with nuclear substrate proteins.

F. MEKs Upstream of the SAPKs, p38s, and ERK5

1. Activation of the SAPKs by SEK1 and MKK7

The SAPKs are activated upon concomitant phosphorylation at Thr-183 and Tyr-185. The MEK SAPK/ERK kinase-1 (SEK1, also called MAPK-kinase-4, MKK4; MEK4; JNK kinase-1, JNK1; and SAPK-kinase-1, SKK1, Table 2) was cloned independently by two groups who employed degenerate PCR to identify novel MAPK signaling components. Two potential initiation methionines, separated by 34 amino acids, are present in the SEK1 cDNA sequence, raising the possibility that two SEK1 isoforms exist that differ at their extreme NH2 termini. Alternative splicing isoforms of SEK1 have not been identified in vivo; however, deletion of the NH2-terminal 34 amino acids of SEK1 could affect its scaffolding function and its regulation by the MAP3K MEK kinase-1 (MEKK1) due to loss of a MEKK1 interaction motif and a basic amino acid-rich SAPK binding site (see sects. E3 and C4) (69, 266, 301, 342).

The homology shared by SEK1 and MEKs-1 and -2 (as well as yeast MEKs) indicated that this kinase lay upstream of MAPKs. It was shown subsequently that SEK1 could phosphorylate and activate all three SAPK isoforms in vivo and in vitro (69, 266). Dérjard et al. (69) also showed that SEK1 could phosphorylate and activate p38 in vivo, when overexpressed, and in vitro (69). However, the significance of p38 activation by SEK1 is unclear. Targeted disruption of sekI in mice has no effect on p38 activity in ES cells (226). Moreover, if SEK1 concentrations in in vitro assays are adjusted to initial rate condi-
tions for SAPK activation, little or no p38 activation is observed (60, 205).

While the identification of SEK1 was encouraging, much biochemical evidence indicated that SEK1 was not the only SAPK-activating MEK. These studies revealed that the spectrum of SAPK activators recruited by different stimuli depended on the stimulus used and on the cell type. Thus fractionation on hydroxylapatite columns of extracts of osmotically shocked 3Y1 fibroblasts demonstrated a broad peak of SAPK activating activity that was fully resolved from a separate peak of SEK1 immunoreactivity (216). Likewise, Mono-S chromatography of KB cell extracts showed that IL-1 failed to activate SEK1, but stimulated a broadly eluting peak of SAPK activating activity distinct from SEK1. Similar multiple peaks of SAPK activating activity were observed in extracts of PC-12 cells treated with arsenite or osmotic shock and in KB cells treated with osmotic shock, UV radiation, or anisomycin (205). In osmotically shocked 3Y1 fibroblasts, SEK1 represented a comparatively minor peak of SAPK activating activity (216). In contrast, SEK1 was more strongly activated by osmotic shock, UV radiation, and arsenite in PC-12 cells (205). In KB cells, SEK1 and other SAPK activators were activated by anisomycin, osmotic shock, and UV radiation (205).

Genetic studies lent further support the contention that multiple SAPK-specific MEKs existed. Thus targeted disruption of sek1, while embryonically lethal, results in only a partial ablation of SAPK activation; sek1−/− ES cells are refractile to anisomycin and heat shock activation of SAPK, while osmotic shock and UV activation of SAPK are unaffected (226). Finally, hemipterous is a Drosophila MEK required for dorsal closure during embryogenesis, and deletion/mutagenesis of hemipterous is lethal (see sect. vC1). Although hemipterous is significantly homologous to SEK1, SEK1 cannot rescue Drosophila mutants wherein hemipterous is deficient (105, 124).

MKK7 (also called MEK7, JNKK2, and SKK4, Table 2) was isolated contemporaneously by several laboratories. Two of the strategies used, database mining or degenerate PCR, sought mammalian MEKs with close homology to hemipterous (91, 188, 218, 314, 340, 353). Alternatively, MKK7 was cloned in a two-hybrid screen as a polypeptide that could associate in vivo with MEK1 (124). The significance of this association is unclear. Consistent with its structural homology to hemipterous, MKK7 can effectively rescue hemipterous lethality, whereas SEK1 cannot (124). MKK7 displays a strong preference for SAPK, even under conditions of high expression. This contrasts with SEK1 which can, under conditions of high overexpression, activate p38 (69, 91, 188, 218, 314, 340, 353). MKK7 can activate all SAPK isoforms tested equally well (91, 188, 218, 314, 340, 353). MKK7, like SEK1 is subject to alternative hnRNA splicing yielding enzymes with three different NH2 termini (α, β, and γ) and two different COOH termini (types 1 and 2). The β- and γ-isoforms bind directly to SAPKs via an NH2-terminal extension not present in the α-isoforms. Upon overexpression, the α-isoforms exhibit a lower basal activity and higher activation by upstream stimuli (315).

MKK7 is strongly activated by TNF and IL-1, conditions which, at best, induce modest SEK1 activation. In contrast, SEK1 and MKK7 are activated with equal potency by osmotic shock while MKK7 is more weakly activated by anisomycin than is SEK1 (91, 188, 218, 314, 340, 353). Thus it is plausible to argue that MKK7 represents at least a substantial portion of the biochemically detected SAPK activating activity present in 3Y1, PC-12, or KB cells subjected to osmotic shock or anisomycin, or in KB cells treated with IL-1.

Although the activation patterns of SEK1 and MKK7 appear not to overlap entirely, there is evidence that SEK1 and MKK7 actively cooperate in the activation of SAPK. Thus, whereas SEK/MKK4 is not strongly activated by TNF, TNF cannot activate SAPK in fibroblasts derived from sek1−/− early mouse embryos, in spite of the fact that MKK7 is expressed in these cells (102, 228). Evidence for the basis of this difference comes from biochemical studies of the mechanisms of SEK1 and MKK7 catalysis. As was mentioned above, SEK1 preferentially targets SAPK Tyr-185 and only weakly phosphorylates Thr-183 (171, 266). In contrast, MKK7 preferentially phosphorylates SAPKs at Thr-183 rather than Tyr-185 (171). Accordingly, when employed individually at low concentrations, SEK1 and MKK7 are comparatively poor SAPK activators in vitro, each stimulating at best a 5- to 10-fold activation of SAPK. When added together, however, SEK1 and MKK7 synergistically activate SAPK, resulting in ~100-fold activation accompanied by equal phosphorylation of SAPK Thr-183 and Tyr-185 (171) in vitro. In vivo, activation of SAPK by TNF, for example, may involve the combined effects of modestly activated SEK1 acting primarily on SAPK Tyr-185 and strongly activated MKK7 acting primarily on Thr-183. Because phosphorylation of both Thr-183 and Tyr-185 is required for full SAPK activation, deletion of sek1 would, by decreasing sharply SAPK Tyr phosphorylation, substantially compromise SAPK activation by TNF (171).

2. Activation of the p38s by MKK3 and MKK6

MKK3 (also called MEK3 and SKK2) and MKK6 (also called MEK6 and SKK3, Table 2) were cloned by degenerate PCR using conserved MEK sequences as templates (59, 69, 248). Both enzymes are highly selective for p38 and do not activate SAPK or ERK under all conditions tested (59, 60, 69, 248). MKK3 and MKK6 differ more
substantially in their substrate selectivity with regard to p38 isoforms than do SEK1 and MKK7. MKK3 preferentially activates p38α and p38β while MKK6 can activate strongly all known p38 isoforms. Similarly, MKK3 appears to be more restricted with regard to activation by upstream stimuli. Whereas MKK6 is activated by all known p38 activators, MKK3, like SEK1, is more strongly activated by physical and chemical stresses (59, 60).

3. Activation of ERK5 by MEK5

MEK5 (Table 2) was cloned by degenerate PCR using conserved MEK sequences as primers (83, 367). Four MEK5 polypeptides arise from differential hnRNA splicing. Splicing near the 5'-end shifts the reading frame and generates long (450 amino acid) and short (359 amino acid) polypeptides. The long polypeptide is localized with the particulate, cytoskeletal fraction while the shorter polypeptide is cytosolic. A second splicing event substitutes two 10-amino acid cassettes in a region between subdomains IX and XI and gives rise to α- and β-MEK5 isoforms analogous to type 1 and type 2 SAPKs (Table 1) (83, 111, 166). This region is important to substrate binding, and, accordingly, α- and β-MEK5s may differ in their substrate specificities. ERK5 was isolated in a two-hybrid screen to identify interactors with MEK5 and is the only known MEK5 substrate (38, 44, 367). Thus it was recently demonstrated that upon expression with the MAP3Ks Tpl-2 and MEKK3 (see sect. II G 1), MEK5 is activated and can itself activate ERK5 (38, 44).

G. Several Divergent Families of MAP3Ks Are Upstream of the SAPKs, p38s, and ERK5

1. General considerations

As with all MEKs, the stress-activated MEKs are regulated by Ser/Thr phosphorylation within a conserved region the P-activation loop of subdomain VIII of the kinase domain. The SEK1 phosphorylation sites are Ser-257 and Thr-261 (69, 266), those for MKK7 are Ser-206 and Thr-210 (α-isoforms) (81, 188, 218, 314, 315, 340, 353), those for MKK3 are Ser-189 and Thr-193 (266), those for MKK6 are Ser-207 and Thr-211 (59, 248), and those for MEK5 are Ser-311 and Thr-315 (for the long form of MEK5) (83, 367).

The number, diversity, and complex enzymology of Ser/Thr kinases that act as MAP3Ks upstream of the stress-activated MAPKs are daunting. This heterogeneity is consistent with the multiple different stimuli that recruit these MAPK pathways. The MAP3Ks upstream of SAPK and p38 fall into three broad protein kinase families: the MEK kinases (MEKKs), the mixed lineage kinases (MLKs), and the thousand and one kinases (TAOs) (Fig. 5).

2. The MEKKs

The MEKKs are the most diverse of eukaryotic MAP3Ks, in terms of enzymology, regulation, and molecular structure. The common feature of the MEKKs is a conserved catalytic domain homologous to that of S. cerevisiae STE11, a MAP3K that regulates both the yeast mating pheromone and osmosensing pathways. Outside of the kinase domains, there is little or no conservation, and these enzymes can, in some instances, catalyze the activation of multiple different MAPK pathways and interact with a wide array of putative regulatory proteins. Mammalian MEKKs include MEKKs-1–4, apoptosis signal-regulating kinase-1 (ASK1), TGF-β-activated kinase-1 (TAK1), and Tpl-2 (the product of the cot protooncogene) (21, 32, 81, 103, 120, 134, 170, 232, 264, 297, 326, 344, 346) (Table 3). NF-κB-inducing kinase (NIK) is an additional MEKK that is a specific activator of the NF-κB pathway (191) and is not discussed here (for review, see Refs. 258, 360).

A) MEKK1, A COMPARATIVELY SELECTIVE ACTIVATOR OF THE SAPK PATHWAY. Johnson and colleagues (170), in an effort to identify MAP3Ks that were upstream of the ERKs, employed a cloning strategy that exploited the existing
knowledge of yeast signaling pathways (the mating factor pathway of *S. cerevisiae* in particular) to isolate ~80-kDa fragment of MEKK1 (Table 3). Thus degenerate PCR primers based on conserved elements of the STE11 sequence and the related *S. pombe* MAP3K byr2 were used to amplify mammalian homologs of the yeast kinases (120, 170). Full-length MEKK1 is a ~160-kDa polypeptide that consists of a COOH-terminal kinase domain (amino acids 1221–1493) and an extensive NH2-terminal domain (amino acids 1–1221) that contains two proline-rich segments (amino acids 74–149 and 233–291) containing putative binding sites for proteins with SH3 domains, a consensus binding site for 14–3-3 proteins (amino acids 239–243), two PH domains (amino acids 439–455 and 643–750), and an acid-rich motif (amino acids 817–1221) that contains two sites for cleavage by cysteine proteases of the proapoptotic caspase family (Asp-871, Asp-874) (30, 90, 170, 344). Within the kinase domain is a binding site for Ras the exact location of which has not been clearly defined (260). MEKK1 can also bind Rho family GTPases (89) (see sect. IVB1).

The 80-kDa MEKK1 fragment first isolated (amino acids 817–1493) was initially shown in overexpression experiments to activate the ERKs. Of note, relative to Raf-1, much larger amounts of MEKK1 were required for MEK1/2 activation. On the basis of these results, it was proposed that MEKK1 was part of a distinct ERK activation mechanism (170). Inasmuch as Ste11p is recruited in a proapoptotic and endogenous SAPK pathway; ERK activation was not observed until massive overexpression of MEKK1 was achieved (347). Similar results were obtained from transient transfection experiments if MEKK1 expression was titrated carefully beginning at low levels of plasmid (210).

Accordingly, in vivo, MEKK1 is a highly selective activator of the SAPKs (Fig. 5). This phenomenon is consistent with biochemical studies that indicate that MEKK1 can activate SEK1 in vitro and in vivo (347). MEKK1 can also activate MKK7α1, α2, β1, and β2 as well as γ1 in vivo, and in transfection experiments, of the MAP3Ks tested, MEKK1 is the most potent overall in vivo MKK7 activator yielding MKK7 activation that is comparable to SEK1 activation (315). However, careful in vitro biochemical and kinetic analysis of MEKK1 activation of MKK7γ1 versus SEK1 indicate that SEK1 is a preferred MEKK1 substrate (other MKK7 isoforms were not tested). Whereas overexpressed MEKK1 can activate the ERKs and even p38, kinetic studies of MEK1/2 and MKK3/6 activation by MEKK1 suggest that these MEKs are activated with a *K* ~cat ~ at least three orders of magnitude lower that that for activation of SEK1 and two orders of magnitude lower than that for activation of MKK7γ1 (342). The basis for this difference in in vivo and in vitro activity is unclear and may reflect artifacts of transient expression. Alternatively, in vivo yet to be identified scaffold proteins (JSAP1/JIP3 is a possible candidate, see sect. IVC2), not employed in in vitro assays using purified proteins, may enable significant MKK7 activation by MEKK1 in vivo. The in vitro selectivity of MEKK1 for SEK1 may be due to the intrinsic scaffold properties of SEK1 (342) (see sect. IVC4). Few extracellular stimuli that activate MEKK1 are known. MEKK1 is significantly activated by TNF (13), microtubule poisons (359), oxidant stress (209), and some receptor Tyr kinase agonists (89).

### Table 3. MAP3K nomenclature

<table>
<thead>
<tr>
<th>Name</th>
<th>Alternate Names</th>
<th>Substrates/Effectors</th>
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<td>MEK1, MEK2</td>
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<td>(MAPKK5)</td>
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### 1) MEKK2 AND -3, MORE PROMISCUOUS MAP3KS

MEKK2 and -3 can activate the SAPKs, p38s, and the ERKs. MEKK3 can also activate ERK5. Using the same approach as that employed in the cloning of MEKK1, Johnson et al. (142) cloned murine MEKK2 and -3 (Table 3). Independently, human MEKK3 was cloned by differential display screening (21, 81). Although these enzymes are clearly *STE11* homologs, as is MEKK1, MEKK2 and -3 are more closely related within their kinase domains to each other (>90% identity) than they are to MEKK1 (~65% identity). MEKK2 and MEKK3 are smaller polypeptides than MEKK1 (~70 and 71 kDa, respectively). Both kinases have a COOH-terminal catalytic domain (amino acids 362–619 for...
MEKK2, amino acids 368–626 for MEKK3) preceded by NH2-terminal noncatalytic domains. The NH2-terminal noncatalytic portions of MEKK-2 and -3 are also significantly similar; however, these regions contain no motifs suggestive of function or regulation (21, 81).

In contrast to MEKK1, which is strongly selective for the SAPKs, MEKK-2 and -3 are considerably more promiscuous. In transfection experiments, each can activate both the SAPK and ERK pathways with nearly equal potency (Fig. 5). Consistent with concomitant ERK and SAPK activation, MEKK-2 and -3 can each activate MEK1 and SEK1 in vivo and in vitro (21, 81). MEKK3 can also activate MKK7α1 and, to a much lesser extent, MKK7α2, -β1, and -β2 in vivo (64, 315). The reason for the selectivity of MEKK3 for different MK7 isoforms is unclear. Early studies indicated that p38 was not activated by MEKK2; however, subsequent studies have shown that MEKK2 and MEKK3 can activate the p38s in vivo (64, 82, 268, see below), and MEKK3 can activate MK3 and MK6 in vivo and in vitro (64, 82) (Fig. 5). Gene disruption studies have revealed an important role for MEKK2 in T-cell signaling and for MEKK3 in cardiovascular development (see sect. vF).

Recent studies indicate that MEKK2 is activated in T cells upon stimulation with antigen-presenting cells (APCs; see sect. vG). Thus D10 cells, a T lymphocyte line reactive to conalbumin and I-AK, were infected with a recombinant retrovirus harboring a green fluorescent protein (GFP)-MEKK2 construct. The infected cells were treated with CH12. LX cells, a I-AK-positive APC line, that had been preloaded with conalbumin. Under these conditions, the D10 cell GFP-MEKK2 was activated and was shown in live cell fluorescence imaging to translocate to the region of the plasma membrane at the interface with the APCs. In contrast, no such APC-dependent translocation of MEKK1 or MEKK3 was observed (269). MEKK2 activation and translocation were dependent on the APCs being loaded with conalbumin. Under these conditions, the D10 cell GFP-MEKK2 was activated and was shown in live cell fluorescence imaging to translocate to the region of the plasma membrane at the interface with the APCs. In contrast, no such APC-dependent translocation of MEKK1 or MEKK3 was observed (269). MEKK2 activation and translocation were dependent on the APCs being loaded with conalbumin, indicating that MEKK2 activation depended on T-cell receptor (TCR) engagement (268). Interestingly, APC treatment also resulted in activation of D10 cell p38 and ERK, and both ERK and p38 activation by APCs was blocked with kinase-dead (Phe502Leu) GFP-MEKK2. Direct activation of SAPK was not measured in these studies; however, kinase-inactive MEKK2 failed to inhibit activation of SAPK by phorbol ester/calcium ionophore, a treatment that mimics the TCR/CD28 costimulation characteristic of the T cell-APC interaction and activates SAPK (see sect. vF) (268). Thus, although MEKK2 has been shown in overexpression assays to activate coexpressed SAPK (21), MEKK2 expressed in D10 T cells, although activated by TCR engagement, apparently does not activate SAPK (268). The reason for this discrepancy is unclear. It is known that, in contrast to T-cell p38s and ERKs, which are activated by TCR engagement alone, T-cell SAPK activation requires both TCR and CD28 costimulation (or phorbol ester/calcium ionophore) (265, 293), presumably the CH12. LX/D10 T-cell interaction involves costimulation. It is conceivable that, at the lower levels of MEKK2 incurred upon expression from recombinant retroviruses, MEKK2 is a preferential activator of ERK and p38, compared with SAPK. Still, the apparent lack of activation of SAPK by MEKK2 in this system is surprising inasmuch as recent gene knockout studies have shown that the SAPKs are required for TCR/CD28-stimulated release of IL-4 and IL-5 (see sect. vF).

Expression of kinase-dead MEKK2 also weakens the stability of APC-T cell conjugates, and wt-MEKK2 strengthens conjugate formation. This effect has been attributed to “inside-out” signaling in which MEKK2 upregulates integrin expression on the T cells. However, neutralizing antibodies to LFA-1, the principal integrin involved in formation of stable T-cell APC conjugates, abrogates conjugate formation in wt-MEKK2 overexpressing cells to the same extent as in cells expressing vector alone (268). No potential direct upstream activators for MEKK2 have been identified; however, APC activation of ERK and p38 can be reversed by wortmannin, suggesting that MEKK2 activation may be PI 3-kinase dependent (268). The effect of wortmannin on MEKK2 translocation was not determined.

Chao et al. (38) recently demonstrated that MEKK3 could activate MEK5 in vivo and, directly, in vitro; and with MEK5 activation, the ERK5 pathway. Interestingly, in these experiments, full-length MEKK3, upon transient expression, was inactive in vivo or in vitro toward MEK5, and deletion of the NH2-terminal 11 amino acids generated a constitutively active construct. For reasons that are unclear, this phenomenon was not observed in earlier studies of MEKK3 activation of the SAPKs, ERKs, or p38s (21, 38, 64, 81, 82). It is possible that the earlier studies employed higher levels of expression resulting in partial in vivo activation. Thus the NH2-terminal 11 amino acids of MEKK3 may serve a negative regulatory function. Chao et al. (38) also observed that EGF activated MEKK3’s in vitro kinase activity and its ability to recruit ERK5 in vivo (38). Interestingly, a dominant inhibitory form of MEKK3 (Lys391Trp) blocked EGF activation of ERK5 but not ERK1 (38). This is likely due to the fact that Raf-1 is the dominant EGF-activated MAP3K for the ERK pathway (195). Thus MEKK3 is a putative effector for mitogen/Tyr kinase regulation of all known mammalian MAPK pathways (Fig. 5).

c) MEKK4 CAN ACTIVATE BOTH THE SAPKS AND THE P38S. MEKK4 (also called MAP three kinase-1, MTK1, Table 3) was isolated by Johnson et al. (103) employing again a PCR strategy based on degenerate primers derived from STE11 and bry2. Independently, Saito and co-workers (120, 297) cloned MEKK4 as a human cDNA that when expressed could rescue osmosensitive yeast missing the
TAK1 is activated by TGF-β regulatory proteins that bind the TAK1 NH2-terminal regulatory motif, and are essential for coupling TAK1 to upstream signals (see sect. vE) (225, 279). Endogenous TAK1 is activated by TGF-β, IL-1, and TNF. In overexpression experiments, TAK1 can activate both the SAPKs and p38s. In vitro and in vivo, TAK1 can phosphorylate and activate SEK1, MKK3, and MKK6 (217, 225, 281, 346).

E) ASK1 can activate both the SAPKs and the p38s. Apoptosis signal-regulating kinase-1 (ASK1, also called MAPK kinase-kinase-5, MAPKKK5, Table 3) is a sixth MEKK that was cloned by Wang et al. (326) and, independently, by Ichijo et al. (134). Both groups used a PCR strategy that employed degenerate primers based on conserved elements of Ser/Thr kinase subdomains VI and VIII (134, 326). ASK1 is a ~150-kDa polypeptide with a centrally located kinase domain (amino acids 677–936), an NH2-terminal extension (amino acids 1–676) that includes segments that bind polypeptides of the TNFR-associated factor (TRAF) family as well as the Fas-associated adapter protein Daxx, and the redox sensing enzyme thioredoxin (see sect. vD) (36, 134, 182, 228, 263, 266). In addition, ASK1 possesses a COOH-terminal extension (amino acids 937–1375) that has also been implicated in TRAF binding (182, 228). ASK1 can activate the SAPKs (via SEK1) and the p38s (via MKK3 and -6) in vivo and in vitro (134, 326) (Fig. 5). Endogenous ASK1 is activated by oxidant stress, TNF, and Fas, and there is evidence that activation of ASK1 by TNF is dependent on TNF-induced generation of reactive oxygen species (108, 134, 182, 228, 263). ASK1 is likely to be an important effector coupling these agonists to the SAPKs and p38s.

Under low serum conditions, expression of ASK1 from a Zn-inducible promoter induces apoptosis in several cell lines (134). The targets recruited by ASK1 (especially if the SAPKs or p38s are involved) that promote apoptosis are unknown. Dominant inhibitory, kinase-dead ASK1 mutants can block apoptosis stimulated by TNF or oxidant stress (134, 263). Thus either ASK1 is a direct apoptogenic signaling component recruited by these ligands, or the dominant inhibitory ASK1 is sequestering a common upstream element that regulates both ASK1 and TNF/oxidant-induced apoptosis.

F) TPL-2 can activate the SAPK, p38, ERKs1/2, and ERK5. Tumor progression locus-2 (Tpl-2) is a rat oncogene that was identified by Tsichlis and colleagues (232) as a homolog of the human protooncogene cot (Table 3). The oncogenic potential of Tpl-2 is activated in rat thymomas as a result of the additive, spontaneous proviral insertion of the Moloney leukemia virus into the infected cell genome at the Tpl-2 locus, the consequence of which is positive selection of tumor cells and enhanced tumor progression in vivo (232). Tpl-2 is a ~50-kDa protein Ser/Thr kinase with an NH2-terminal domain, of unknown function, which is truncated in some mRNA splicing isoforms, a central catalytic domain (amino acids 139–394) that is significantly homologous to the S. cerevisiae MAP3K STE11, and a COOH-terminal regulatory domain (amino acids 395–467) (32, 232). Moloney leukemia virus proviral insertions at Tpl-2 always target the last intron of the gene and elicit the enhanced expression of a COOH-
terminally truncated protein, both through enhanced transcription and mRNA stabilization. It is likely, therefore, that the COOH-terminal regulatory domain of Tpl-2 exerts a negative effect on Tpl-2 activity. In support of this idea, the free Tpl-2 kinase and COOH-terminal domains, when expressed together in Sf9 cells, interact in vivo, as demonstrated in communoprecipitation experiments. Moreover, transgenic mice expressing the COOH-terminally truncated protein develop T-cell lymphomas, whereas expression of the wild-type protein is without effect (32).

Tschilis and colleagues demonstrated that transient expression of Tpl-2 activated the ERK pathway in parallel to Raf-1, and possibly downstream of Ras (233). Subsequently, Ley and co-workers (264) as well as Tschilis and co-workers (32) showed that expression of Tpl-2 activated the SAPKs and ERKs with equal potency. Ley and co-workers (264) then demonstrated that, consistent with its homology to STE11, Tpl-2 was a MAP3K that could directly activate MEK1 and SEK1 in vivo and in vitro (Fig. 5). While overexpression of full-length Tpl-2 activates both the SAPKs and ERKs, expression of the oncoogenic COOH-terminally truncated Tpl-2 results in substantially greater SAPK and ERK activation, further supporting the contention that the COOH-terminal domain negatively regulates Tpl-2 activity (32).

A recent study by Chiariello et al. (44) revealed a far broader substrate specificity for Tpl-2. Thus transient expression of Tpl-2 induces c-jun transcription and transformation of NIH3T3 cells. c-Jun expression is required for Tpl-2 transformation inasmuch as TAM67, a dominant inhibitory mutant c-Jun construct wherein the δ-domain and phosphoacceptor sites have been deleted, strongly blocks Tpl-2, but not v-Ras transformation (44). Insight into the mechanism by which Tpl-2 recruits the c-jun promoter came from studies employing the SAPK binding portion of the scaffold protein JNK-interacting protein-1 (JIP1, see sect. III), a potent and specific inhibitor of SAPK-dependent signaling. Tpl-2 induction of c-jun is dependent on activation of the ERK5/MEF2C pathway as well as on the SAPKs. Thus expression of the JIP1 SAPK binding pocket blocked MEKK1 induction of a c-jun-Luc reporter completely, while only partially blocking Tpl-2 induction of c-jun-Luc. Promoter bashing experiments indicated that MEKK1, Tpl-2, and Ser311Asp/Thr315Asp (constitutively active, DD-MEK5) MEK5 could induce c-jun-Luc, and deletion of the AP-1 site crippled MEKK1 and, to a slightly lesser extent, Tpl-2 trans-activation of c-jun-Luc. In contrast, deletion of the MEF2C site on the c-jun reporter crippled completely Tpl-2 and DD-MEK5 induction of c-jun-Luc without affecting MEKK1 induction of c-jun-Luc. SAPK-dependent and -independent trans-activation of c-jun-Luc was further demonstrated with DN constructs; thus Tpl-2 transactivation is blocked with DN MEK5, MKK6, and ERK as well as expression of JIP1. All of these DN constructs are shown to be acting specifically (44). Consistent with this, ectopic expression of Tpl-2 expression activated ERK1, SAPK (γ), p38γ, and ERK5 but not p38α or δ. Correspondingly, coexpression with Cot activated MEK1, MKK6, MEK5, and SEK1. The selective activation of p38γ was curious, insofar as MKK6 can activate all known p38 isoforms in vitro and, in transfection experiments in vivo (44). Mechanisms of sequestration, such as scaffold proteins, may isolate Tpl-2 and MKK6 from all p38 isoforms other than γ. In correspondence with the broad effector specificity of Tpl-2, Tpl-2-dependent transformation requires p38, SAPK, and ERK5. Thus Tpl-2-induced NIH3T3 cell focus formation is completely blocked by dominant inhibitory MEK5, MKK6, or JIP1 (44). Extracellular stimuli that recruit Tpl-2 in vivo are unknown.

3. The mixed lineage kinases: MLK3, MLK2, and DLK selectively activate the SAPKs

The mixed lineage kinases (MLKs) are a small family of protein Ser/Thr kinases that share a general structural configuration wherein an NH2-terminal kinase domain is followed by one to two leucine zippers, a Cdc42/Rac interaction and binding (CRIB) domain (see sect. III), and a COOH-terminal proline-rich domain with several consensus SH3 binding motifs. Four MLKs have been identified: MLK1; MLK2 (also called MKN28 cell-derived Ser/Thr kinase, MST, Table 3); MLK3 (also called SH3 domain-containing proline-rich kinase, SPRK, or protein Tyr kinase-1, PTK1); and dual leucine zipper kinase (DLK, also called MAPK upstream kinase, MUK, or zipper-containing protein kinase, ZPK, Table 3). MLK2 and MLK3, in addition to the common features described above, contain SH3 domains NH2-terminal to the kinase domains (78, 101, 127).

Although the MLKs are clearly Ser/Thr specific in vitro and in vivo, as their names suggest, the kinase domains of the MLKs bear structural similarities to both Ser/Thr and Tyr kinases. Thus, for example, MLK1 contains a Lys residue (Lys-129 in the sequence His-Arg-Asp-Leu-Lys) in subdomain VIb that is characteristic of Ser/Thr kinases; however, two Trp residues in MLK1 subdomain IX (Trp-192 and Trp-199) are highly conserved among Tyr kinases, as is a motif in subdomain XI (Met-241-Glu-Asp-Cys-Trp-Asn-Pro-Asp-Pro-Arg-Pro-Arg-Pro-Phe-255) which conforms to a conserved region in Tyr kinases (Met-X-X-Cys-Trp-X-X-Asp/Glu-Pro-X-X-Arg-Pro-Thr-Phe, where X is any amino acid) (78).

Three of the four known MLKs have been assayed for activation of MAPK pathways: MLK3, MLK2, and DLK. All three are potent activators of the SAPKs in vivo in transfection experiments (88, 121, 122, 249, 315). In contrast to the generally more promiscuous MEKKs, the MLKs are entirely SAPK pathway specific. ERK, p38, and NF-κB are not activated by MLK-2, -3, or DLK, except under condi-
tions of extreme overexpression (Fig. 5). MLK3, MLK2, and DLK are all established MAP3Ks. Thus, in vitro and in vivo, MLK3, MLK2, and DLK can activate SEK1 to a degree commensurate with that catalyzed by MEKK1 (121, 122, 249, 309, 315). In addition, MLK3 and DLK can activate MKK7 in vivo in transfection experiments, and in vitro, albeit with differential isoform selectivity in vivo. Thus, in both instances, MKK7β1 and -β2 are strongly activated in vivo while α-isosforms are modestly activated (315). Although this selectivity is likely due to the structural differences in the NH₂ termini of the α- and β-isosforms of MKK7 (discussed in sect. uFI), which, in turn, may affect interactions with MLK3 and/or scaffold proteins such as JNK interacting proteins (JIPs) or JNK/SAPK interacting protein-1 (JSAP1/JIP3, sectionIIIC), the significance and molecular basis of this selectivity are unknown. MLK2 can also activate MKK7α1. Indeed, MLK2 is a stronger MKK7α1 kinase than it is a SEK1 kinase (122, 315).

4. TAO kinases are novel MAP3Ks that regulate the p38s and are structurally homologous to both Ste20 and MAP3Ks

Cobb and colleagues (133) recently identified the TAO kinases (TAO1 and TAO2), a novel family of 1,001 amino acid Ser/Thr kinases, as part of a scheme to identify additional mammalian kinases homologous to S. cerevisiae STE20, a proximal kinase thought to be important in the regulation of the Ste11p >Ste7p >Fus3p/Kss1p yeast mating pheromone MAPK core signaling module (42, 120, 133). A possible splicing isoform of TAO2, referred to as prostate-derived STE20-like kinase (PSK, Table 3), that includes an extended COOH-terminal tail, but is otherwise identical to TAO2, was cloned independently by Moore et al. (214) as a kinase expressed at elevated levels in prostate tumors. TAOs consist of NH₂-terminal kinase domains and very large (700 amino acids) COOH-terminal extensions of poorly characterized function. The kinase domains of TAOs are significantly homologous to Ste20p (40% identity) and the GCKs (43% identity with GCK) both established mammalian Ste20-like kinases (42, 120, 133, 163, 214). However, there is appreciable identity with the MLK2 kinase domain (33% overall), especially within the substrate binding motifs (133).

TAO-1 and -2 appear to be specific activators of p38. The purified kinase domain of TAO1 will directly phosphorylate and activate SEK1, MKK3, and MKK6 in vitro; however, in vivo only MKK3 is activated. Moreover, when expressed in Sf9 cells from a recombinant baculovirus, TAO1 will interact in vivo with and coimmunoprecipitate with MKK3. Thus, although TAO1 bears significant homology to Ste20s and GCKs, unlike kinases of the Ste20 and GCK groups, which are thought to regulate MAP3Ks (see sect. n, B3 and G1), TAO1 appears to be a direct MAP3K selective for MKK3 (133) (Fig. 5). The ability of TAO1 to catalyze directly the activation of MEKs may be due to the kinase domain homology with MLK2 in the substrate binding region. The high in vivo selectivity of the TAOs for MKK3 may be due to the presence of a specific MKK3 binding site. Cobb and colleagues (42) identified a region on the TAO2 polypeptide (amino acids 314–451) that selectively binds MKK3 in vitro and in vivo. Interestingly, this region is rich in acidic amino acid residues, similar to the CD loop of MAPks, a region that binds to basic MAPK interaction motifs on MAPK regulators and substrates (Fig. 5D, sect. uE3) (301). Thus an analogous acidic amino acid-rich/basic amino acid-rich interaction may govern the TAO-MKK3 interaction. It will be important to determine if the NH₂-terminal basic MAPK interaction motif on MKK3 (301) is important for binding TAOs. A similar acidic amino acid-rich site is present in TAO1 (133), although its binding to MKK3 was not tested.

PSK, the TAO2 splicing isoform, appears to activate selectively the SAPK pathway; ERK and p38 are not activated in transfection experiments (214). This difference in pathway selectivity is curious given that the MKK3 interaction loop found in TAO2 (42) is present in PSK (214). The COOH-terminal tail unique to PSK may account for the altered specificity of PSK; however, this has not been demonstrated.

III. PUTATIVE ELEMENTS UPSTREAM OF STRESS-ACTIVATED MITOGEN-ACTIVATED PROTEIN KINASE CORE PATHWAYS

A. Regulation of the SAPKs and p38s by Rho Family GTPases

1. General considerations

Sections uB to uG have described much of what we know of mammalian stress-activated MAP3K →MEK →MAPK core signaling modules. The following five sections (sects. uA to uE) will describe some of the known proximal signaling components thought to couple to these core modules.

Monomeric GTPases of the Ras superfamily are potent upstream activators of signal transduction pathways, and Ras is pivotal to the activation of the ERK pathway. The activation of Ras by guanine nucleotide exchange factors (GEFs) and the inactivation of Ras by GTPase activating proteins (GAPs) has been extensively reviewed (23, 201, 202). Ras regulates ERK predominantly through triggering the activation of the MAP3K Raf-1 (195). The regulation of Raf-1 by Ras is a paradigm for MAP3K regulation, and studies of Raf-1 regulation point to three crucial events that govern MAP3K activation: binding to an upstream activating protein accompanied by translocation to the membrane, phosphorylation, and homol-
gomerization. Thus Raf activation requires binding to GTP-Ras, an event that results in membrane translocation (195). At the membrane, Raf is phosphorylated by several protein kinases including Tyr kinases such as Src and Ser/Thr kinases such as p21-activated kinase (PAK)-3, an effector for Rac1, a Rho family GTPase that is itself a Ras effector (87, 118, 160, 198). Phosphorylation of Raf-1 results in structural alterations that change the binding to proteins of the 14–3-3 family. These structural changes are necessary for Raf-1 activation (320). Ras-dependent oligomerization is also necessary for Raf-1 activation (190). In the same vein, activator protein binding/membrane translocation, phosphorylation, and oligomerization may be important in the regulation of stress-activated MAP3Ks.

Whereas Ras itself can directly influence the ERK pathway by recruiting Raf-1, at least some stress-activated MAPK core pathways may be regulated by members of the Rho subfamily of the Ras superfamily. The Rho subfamily is comprised of mammals of the Rho (RhoA-E), Rac (Rac1 and -2), and Cdc42 (Cdc42Hs, G25K, Tc10, and Chp) groups (8, 23, 114). As with other members of the Ras superfamily, Rho subfamily GTPases are active in the GTP-bound state (promoted by GEFs) and inactive in the GDP-bound state (promoted by GAPs) (23, 201, 202). Rac, Cdc42Hs, and other Rho subfamily GTPases share a large number of GEFs, belonging to the Dbl family, including Ost, a GEF for RhoA and Cdc42Hs; Tiam-1, a GEF for Rac1 and Cdc42Hs; Lbc, a GEF for RhoA; and facio-gential dysplasia-1 (FGD1), a GEF selective for Cdc42Hs (33).

All Rho family GEFs consist of a conserved Dbl homology (DH) domain that is necessary for promoting GDP dissociation and a plekstrin homology (PH) domain. Inositol phospholipids, including inositol 3,4,5-trisphosphate, the product of PI 3-kinase activity, are putative targeting signals for PH domain-containing polypeptides (79). Activation of PI 3-kinase requires Ras (255) and, accordingly, Rho family GEFs themselves may be subject to regulation by Ras → PI 3-kinase (33, 118). Indeed, mitogen activation of Rac1 requires activation of PI 3-kinase (118).

Among the first recognized physiological functions for the Rho family was regulation of the actin cytoskeleton (33, 114). Thus fibroblasts in culture will form actin stress fibers that associate with the focal adhesions which, in turn, bind the cells to the substratum. Formation of these stress fibers requires RhoA (114). Within minutes of mitogen treatment of cells, transient changes in the actin cytoskeleton occur. Notably, any dense perinuclear actin filaments present in the resting cells are disassembled, and the cells form peripheral filopodia (actin microspikes) and lamellosplodium (membrane ruffles) from the free actin (114). Filopodium formation requires Cdc42Hs. Lamellosplodium formation requires Rac1 (114).

The physiological functions of lamellosplodium and filopodia, with regard to the control of cellular proliferation and differentiation, are incompletely understood; however, the ability of Rho family GTPases to trigger these cytoskeletal changes correlates at least in part with Rho family GTPase transforming capability. Thus Rac, Rho, and Cdc42Hs all appear necessary for the entry of G0 cells into the cell cycle, and expression of constitutively active, GTPase-deficient mutants of Rac, Rho, or Cdc42Hs will drive quiescent cells into the cell cycle in the absence of serum. Conversely, ras transformation is blocked by Asn-17, exchange-deficient mutants of Rac or Cdc42, or by effector loop mutations that also abrogate cytoskeletal functions (114) (Fig. 6A).

Constitutively active, GTPase-deficient (Val-12) mutants of Rac1, Cdc42Hs, or Chp are potent activators of the SAPKs (8, 52, 211, 229). In addition, Val12-Rac1 and Cdc42Hs can activate p38 (12, 364). RhoA generally does not activate SAPK or p38 but can in certain cell types (notably 293 cells) (306). Consistent with these findings, expression of the Dbl protooncogene product or FGD1, Rho GEFs that recruit Rac and Cdc42, also results in strong SAPK activation (52, 366). Transforming alleles of

![FIG. 6. G protein regulation of stress-activated MAP3Ks. A: Ras superfamily regulation of MAP3Ks. B: trimeric G protein regulation of SAPKs.](image-url)
ras and EGF (through Ras) may signal to the SAPKs via Rac1 inasmuch as Asn-17 Rac1 can block the activation of SAPK by these stimuli. In addition, Rac1 is also involved in SAPK activation in response to CD3-CD28 T-cell costimulation (137, 229). Cdc42Hs, in contrast, is activated by lysophosphatidic acid, bombesin, and, in spreading melanoma cells, engagement of chondroitin sulfate proteoglycan (80, 114).

While Rac1 and Cdc42Hs can each regulate both MAPK pathways and the cortical actin cytoskeleton, it appears that these functions are mediated by distinct pathways. Support for this idea comes from the use of effector loop mutants (334). The effector loop of all Ras superfamily GTPases lies adjacent to the guanine nucleotide binding site (23, 201, 202). Conformational changes that occur upon GDP-GTP exchange expose the effector loop enabling the binding of target proteins. Point mutations in the effector loops of Ras superfamily proteins can disrupt the binding of selective effector proteins resulting in the selective loss of functions attributable to those proteins that can no longer bind (23, 114, 201, 202, 334). Thus Phe37Ala Rac1 is unable to stimulate membrane ruffling but still activates SAPK. Conversely, neither Tyr40Cys Rac1 nor Cdc42Hs can activate SAPK, but each is still able to induce, respectively, membrane ruffling and filopodium formation (168).

2. Coupling Rho GTPase targets to SAPK and p38 core signaling pathways

As mentioned above, proteins of the Ras superfamily activate their targets in part through a direct binding interaction between the target protein and the effector loop of the GTP-charged G protein (201, 202). This binding results in membrane translocation and, in the case of Ras-Raf, is a necessary prerequisite for subsequent activation events such as homologimerization and phosphorylation by upstream kinases (160, 190, 198). Most, but not all, direct targets for Rac1 and Cdc42Hs contain a so-called Cdc42/Rac interaction and binding (CRIB) domain that interacts directly with the effector loops of Rac1 and Cdc42Hs (27). Several polypeptide species including protein kinases and adapter proteins are candidate Rac1 and Cdc42Hs effectors that couple to the SAPKs and p38s.

A) PAKS. STE20 encodes a S. cerevisiae Ser/Thr protein kinase with an NH₂-terminal regulatory domain containing a CRIB motif that binds Cdc42Sc, the yeast Cdc42Hs ortholog (120). Ste20p is thought to regulate the mating pheromone MAPK core signaling module Ste11p →Ste7p →Fus3p/Kss1p, and genetic studies suggest that Ste20p action is upstream of Ste11p, although the mechanism of Ste20p action in this process has yet to be defined completely (120). Genetic epistasis studies provisionally placing Ste20p upstream of the MAP3K Ste11p led to the notion that Ste20-like kinases in mammals might couple Rho GTPases MAPK core signaling modules (120).

The PAKs (PAK1, also called αPAK; PAK2, also called γPAK or hPAK65; PAK3, also called βPAK; and PAK4, Table 4) are a family of mammalian kinases that are structural and functional orthologs of Ste20p (2, 120, 192, 197, 201, 305). PAK1 and PAK2 were originally purified as protein kinases that selectively bound GTP-Rac1 and GTP-Cdc42Hs (193, 197, 305), and in vitro and in vivo, the kinase activity of PAKs is activated upon binding GTP-Rac1 or GTP-Cdc42Hs through a process that involves an obligatory autophosphorylation event (197). PAK2 can also undergo activation during apoptosis through a mechanism that involves caspase 9-mediated cleavage at Asp-212, a process that removes the NH₂-terminal regulatory domain (including the CRIB motif) (175). The significance of this cleavage-mediated activation is unclear, although PAK2 may mediate some morphological functions associated with apoptosis such as cell shrinkage (175).

A major function of the PAKs (PAK1 in particular) is to serve as effectors for Cdc42 and Rac in the regulation of the actin cytoskeleton (271, 272). It is conceivable that, as is the case with Raf-1 activation by PAK3, stress-activated MAP3Ks might, once translocated to the membrane upon binding GTP-loaded Rho family GTPases, undergo activating phosphorylation catalyzed by PAKs contemporaneously located at the membrane as a consequence of binding GTP-Rac1 or Cdc42. Indeed, several reports have indicated that constitutively activated forms of PAKs can activate coexpressed SAPKs and p38s (12, 25, 236, 364). Thus, for example, mutation of Leu-107 to Phe, within the CRIB motif of human PAK1, results in a constitutively active construct that can activate coexpressed SAPK (25). Moreover, addition to cell-free extracts of Xenopus oocytes, of a PAK1 mutant wherein the NH₂-terminal regulatory domain has been deleted also results in substantial SAPK activation (236). However, SAPK activation by coexpressed PAKs has not been universally observed. Moreover, the activation of SAPK by wild-type PAKs is generally small, and expression of PAKs does not

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<tr>
<th>Name</th>
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<th>Substrates/Effectors</th>
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<tr>
<td>GCK</td>
<td></td>
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<tr>
<td>GCKR</td>
<td>Kinase homologous to Ste20 (KHS)</td>
<td>MEKK1, βMLK3 MEKK1</td>
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<td>GLK1</td>
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<td>HPK1</td>
<td>NIK (not to be confused with NF-κB-inducing kinase), HPK1/GCK-related kinase (HGK)</td>
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TABLE 4. GCK/PAK nomenclature

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synergize with Rac or Cdc42 to activate further SAPK (89). Thus it is not clear if PAKs are true effectors coupling Rho family GTPases to the SAPKs and p38s.

b) MAP3Ks. Several established stress-sensitive MAP3Ks, including MEKK1 (89) (discussed in sect. ivB1), MEKK4 (89, 103) (discussed in sect. ivF1), MLK2, and MLK3 (27, 222) (discussed in sect. ivG2), are putative effectors for Rac1 and Cdc42Hs (Fig. 6A). However, whereas the PAKs are clearly activated by Rac1 and Cdc42Hs, the functional role of the interactions between these MAP3Ks and Rho family GTPases is, in most cases, unclear.

c) PLENTY OF SH3 DOMAINS. Plenty of SH3 domains (POSH) is a novel ~90-kDa polypeptide that consists of four SH3 domains (amino acids 139–190, 198–254, 457–511, and 838–892), a domain rich in putative polyproline SH3 binding sites (amino acids 368–405), but no CRIB motif. In spite of this, however, POSH interacts directly with Rac1 (but not Cdc42Hs, Ras, or Rho) in yeast two-hybrid assays and a GTP-dependent manner in vitro. The Rac binding domain has been localized to amino acids 292–362, a domain that appears thus far to be unique to POSH (302). Transient expression of POSH in COS1 cells results in potent SAPK activation. In addition, POSH expression triggers nuclear translocation of NF-κB and apoptosis (302). POSH recruitment by Rac1 effector mutants correlates tightly with the ability of these Rac1 mutants to activate the SAPKs. Thus, in contrast to MLK3 and MLK2, POSH can interact with Phe37Ala-Rac1, a Rac1 effector mutant that cannot elicit lamellipodium formation but does activate coexpressed SAPK (168, 302). However, POSH cannot interact with Tyr40Cys Rac1, a second Rac1 effector mutant that does not activate the SAPKs in vivo (168, 302). These results suggest that POSH, but not the MLKs, is a likely effector for Rac1 activation of the SAPKs. However, as is discussed in section ivG2, there is good evidence implicating MLK3 as an effector for Cdc42Hs. The mechanism by which POSH recruits the SAPKs is unclear. It is plausible to speculate that the SH3 domains of POSH may serve a scaffolding function, binding MAP3Ks such as MEKK1 or MLKs, that contain SH3 binding sites.

d) SUMMARY. To summarize, PAKs as well as MEKK-1 (see sect. ivB1) and -4 (see sect. ivF1) can interact with both Rac1 and Cdc42Hs. Constitutively active PAK mutants can, in some but not all instances, modestly activate the SAPKs in vitro; however, kinase-dead MEKKs cannot block this activation, calling into question the original hypothesis, based on studies of yeast signaling (120), that, in response to activated Rho family GTPases, PAKs would recruit MEKKs to activate the SAPKs and p38s. In contrast, MLK3 and MLK2 are likely effectors for Cdc42Hs (but not Rac1) (see sect. ivG1) while POSH is a candidate adapter protein that couples Rac1 to the SAPKs.

B. Regulation of the SAPKs and p38s by Heterotrimeric G Proteins: Implications for Cardiovascular Diseases

A number of ligands that bind to seven pass membrane receptors can strongly recruit the SAPKs. These include potent vasoactive compounds, suggesting a major role for the SAPKs in cardiovascular regulation. Thus ANG II and endothelin-1 are strong SAPK agonists in several cell types including cardiomyocytes (ANG II and endothelin-1), hepatocellular carcinomas (ANG II), and pulmonary epithelia (endothelin-1) (46, 92, 275, 369). In addition, the SAPKs can be activated upon engagement of the m1 muscarinic acetylcholine receptor and the thrombin receptor (51, 92).

The α- and βγ-subunits of heterotrimeric G proteins couple seven pass receptors to intracellular effectors. Trimeric G protein α-subunits are divided into four subfamilies based on primary sequence conservation and shared effectors: Gαi, Gαq, Gαs, and G12α (23, 115, 154). There are 5 different mammalian β-subunits (β1–5) and 10 different γ-subunits (γ1–10) known (115).

Receptor engagement fosters GTP binding to the α-subunit that results in dissociation of the α-subunit from the βγ-complex. Free α and βγ can each bind and regulate target proteins. Although the subunits of trimeric G proteins do not assemble completely indiscriminately, the sheer number of different α-, β-, and γ-subunits suggests that an extremely diverse array of heterotrimeric G protein complexes can be assembled within a cell (115, 154).

Of the ligands that engage seven pass receptors and activate the SAPKs, not much is known regarding the subset of trimeric G proteins to which these receptors couple. The ANG II and thrombin receptors recruit primarily the Gαi family α-subunit Gαi and possibly the G12α family α-subunits G12α and G13α (115).

The ability of some Gα subunits to transform cells and activate the ERKs (100), coupled with the observation that ANG II, endothelin I, and other ligands that bind to trimeric G protein-coupled receptors, could recruit the SAPKs (92, 275, 369), led to the examination of trimeric G protein subunits as SAPK activators. From these studies, it is evident that G protein α- and βγ-subunits can both recruit the SAPKs and that the degree of activation as well as the mechanism (α- or βγ-driven) are cell and stimulus dependent.

Thus transient overexpression of GTPase-deficient mutants of the G12α family α-subunits G12α and G12β, or the Gαi family α-subunits G12α or Gαi results in modest (PC12 cells) to strong (293 cells) SAPK activation. Strong activation of SAPK is also observed in NIH3T3 cells transformed upon stable overexpression of GTPase-deficient G12 and G13α (50, 119, 243). p38 was not tested in these studies (Fig. 6B).
Activation of SAPK by these constitutively active G protein α-subunits has not been universally observed, and it is evident that G protein β- and γ-subunits can also activate coexpressed SAPK and may, in fact, represent a major mechanism by which trimeric G proteins signal to the SAPKs (53). For example, in one study of COS-7 cells, SAPK is weakly activated upon transient coexpression with GTPase-deficient G_{12α}, G_{12α}, or G_{13α}, and trimeric G protein signaling to the SAPKs may be mediated by βγ-subunits. Coexpression in COS-7 cells of G_β, with G_γ results in much stronger SAPK activation (53). Furthermore, recruitment of SAPK by the m1 or m2 muscarinic acetylcholine receptor can be reversed with a construct expressing the G protein receptor-binding domain of the β-adrenergic receptor kinase (β-ARK), a scavenger for free G_{βγ} subunits (53). Weaker α-subunit activation of the SAPKs may not be a general feature of all COS cells (see below), and, as noted above, other studies using different cell types have observed substantial SAPK activation upon coexpression with G_{12α} or G_{13α} (50, 243).

Several candidate mechanisms have been proposed for the coupling of heterotrimeric G proteins to the SAPKs; however, there is little really definitive evidence in favor of any of these models (Fig. 6B). Inasmuch as Asn-17 Ras or Rac blocks G_{12α} activation of SAPK, a role for Ras and Rac has been proposed for G_{12α} activation of SAPK. The role of Ras as an effector for G_{12α} may be cell dependent, however. Thus Asn17-Ras can block signaling from GTPase-deficient Gln229Leu G_{12α} to SAPK in NIH3T3 cells and in stably transfected COS cells but not in HEK293 cells (50, 243). In contrast, Asn17-Rac can inhibit Gln229Leu-G_{12α} activation of SAPK in both NIH3T3 and HEK293 cells (50, 243). Moreover, Asn17-Rac can also inhibit activation of SAPK by free G_{βγ} (53). G_{βγ} signaling, at least in COS cells, may also require Ras given that Asn17-Ras blocks SAPK activation by Gln226Leu GTPase-deficient G_{12α} (50).

Nonreceptor Tyr kinases may also couple trimeric G proteins to the SAPKs. Thus the Bruton’s Tyr kinase (Btk) directly binds and is activated by G_{12α} (141), a putative effector of the receptor for ANG II, a good activator of the SAPKs (115, 369). Ectopic overexpression of Btk also activates coexpressed SAPK (153). The mechanism by which G_{12α} binding activates Btk is unclear. Btk has a PH domain and, once recruited to the membrane by G_{12α} may be an effector for PI 3-kinases, downstream of Ras, trimeric G proteins that recruit Ras or trimeric G protein βγ-subunits themselves (Fig. 6B). G_{α} and free G_{βγ} subunits can also activate phospholipase C-β (PLC-β). Inositol trisphosphate generated as a consequence of PLC-β activity releases intracellular Ca^{2+} stores into the cytosol (115). Pyk2 is a nonreceptor Tyr kinase that is activated in vivo by elevations in intracellular free Ca^{2+} and in some cells (hepatocellular carcinoma cells) by ANG II (75, 369). Pyk2, acting in concert with Src, has been implicated in the relay of signals from trimeric G proteins to ERKs, and transient expression of Pyk2 and SAPK also results in significant SAPK activation (75, 312). Thus agonists such as ANG II could recruit the SAPKs by direct G_{α}-mediated PLC-β/Ca^{2+}-dependent activation of Pyk2 (Fig. 6B). It is attractive to speculate that Btk and Pyk2 recruit Ras and, possibly, Rac1, thereby triggering activation of SAPK; however, data in support of this hypothesis are not yet available. PLC-β activation also results in the generation of diacylglycerol and activation of classical PKCs; however, evidence implicating classical PKCs in coupling the SAPKs or p38s to seven pass receptors is unavailable (165, 166).

C. Regulation of the SAPKs by Scaffold Proteins

Given the large number of MAP3Ks and additional upstream regulators of the SAPKs and p38s, the organization of individual MAP3K → MEK → MAPK core signaling components into groups subject to regulation by select activators, and able to recruit select effectors, is vital to the maintenance of signaling specificity, efficiency, and integrity. In lower eukaryotes, this sequestration is mediated by scaffolding proteins that collect specific MAP3K → MEK → MAPK core modules into organized clusters (120, 242). These scaffolding proteins may be distinct polypeptides such as the S. cerevisiae scaffold protein Ste5p (Fig. 7A), or, alternatively, as with the yeast MEK Pbs2p, the signaling components themselves may, in addition to signaling activity, possess intrinsic scaffold properties (120, 242) (Fig. 7B).

Scaffold proteins for mammalian stress-activated MAPK pathways are now being identified. As with yeast, these include proteins whose sole apparent function is that of a scaffold, others which are multipurpose binding proteins, and still others which are also components of MAP3K → MEK → MAPK core modules.

1. JIPs: distinct scaffold proteins that may regulate MLK → MKK7 → SAPK core signaling modules

JNK interacting protein-1 (JIP1) is the founding member of a novel family of mammalian scaffold proteins that was identified from a two-hybrid screen that sought polypeptides that could interact with SAPK/JNK1. A ~60-kDa polypeptide, JIP1, consists of an NH₂-terminal domain that binds SAPKs, but not ERKs or p38s (amino acids 143–163), and a COOH-terminal SH3 domain (amino acids 491–600). In between is a proline-rich segment (amino acids 281–448) with several consensus putative SH3 binding sites (72). JIP1 can bind elements at all three dynamic or regulated in any way. MLK3 binds JIP1
via the JIP1 SH3 domain, whereas M KK7 binding to JIP requires a central region (amino acids 283–471) of the JIP1 polypeptide that overlaps with the proline-rich region (72, 336). JIP1 can also bind the mammalian GCK homolog hematopoietic progenitor kinase-1 (HPK1) (but not GCK-related, another GCK homolog, see sect. III D) (72, 336). The binding of HPK1 is likely to be indirect, mediated by the binding of HPK1 to MLK3 (see sect. IV G1); however, the ability of JIP1 to potentially link HPK1 to a complete core signaling module fits nicely with the idea of JIP1 as a true scaffold protein.

The exact biological function of JIP1 is unclear, and the binding of endogenous JIP1 to endogenous MAPK signaling components has not been demonstrated; however, coexpression of JIP1 with MLK3 or M KK7 enhances the ability of these proteins to foster SAPK activation in vivo. In contrast, overexpression of JIP1 inhibits activation of SAPK by extracellular stimuli including TNF and UV radiation, and there are no known stimuli whose activation of SAPK is enhanced by JIP expression (336). It is likely that JIP1 serves to organize SAPK core signaling modules so as to permit regulation by selective sets of upstream stimuli. Accordingly, overexpression of JIP1 could nonspecifically sequester endogenous MLK3, DLK, M KK7, or SAPK, enhancing the ability of recombinant, ectopically expressed MLK3, DLK, or M KK7 to activate

FIG. 7. Regulation of the SAPKs and p38s by scaffold proteins. A: distinct scaffold proteins. Yeast Ste5p is the model for lower eukaryotes. B: core elements with scaffold properties. In yeast, this is exemplified by Pbs2p; in mammals by SEK1. In A, we have also illustrated the interactions mediated by SEK1 scaffold function that may occur contemporaneously with regulation by discrete scaffold proteins.
the SAPKs, but inhibiting the ability of stimuli that normally recruit these proteins independently of JIP1 to activate the SAPKs. Alternatively, overexpression of JIP1 could disrupt the normal stoichiometry of JIP1 and its effectors. By definition, for a scaffold protein to function appropriately, its level in a cell must be tightly regulated such that it can bind its effectors at an appropriate stoichiometry. Thus, if the concentration of an ectopically expressed scaffold protein such as JIP1 exceeds the concentration of the endogenous signaling components it regulates, a situation arises, the prozone effect, where no single scaffold protein molecule is associated with all of the signaling effectors it must coordinate to regulate its function. At this point, the overexpressed scaffold protein becomes an inhibitor of signaling.

Recently, the cloning of JIP2 was reported. JIP2 is structurally and functionally quite similar to JIP1 and is almost exclusively localized in brain. As with JIP1, JIP2 interacts in vivo with MLK2 and -3 (but not MEKK family MAP3Ks), with MKK7 (but not MEK1, MKK3, SEK1, or MKK6), and with SAPK-α, -β, or -γ (but not p38 and ERK) (355). There are significant differences in the JNK binding properties of JIP1 and JIP2, however. JIP1 binds all SAPK isoforms more strongly than does JIP2, and both JIPs preferentially interact with the SAPK-γ isoform (355). JIP1 and -2 can potentiate (2.5- to 3-fold) activation of SAPKs-α, -β, or -γ by coexpressed MLK3, and the JIP proteins (endogenous and ectopically expressed) can homo- and heterodimerize. Immunofluorescence studies indicate that JIP proteins are localized in the cytoplasm and peripheral membrane, with JIP1 more localized in membrane projections of PC12 pheochromocytoma cells. The significance of this localization is unclear (355).

2. JNK/SAPK associated protein-1/JIP3 is a second SAPK pathway scaffold protein

Murine JNK/SAPK-associated protein-1 (JSAP1) was isolated by Ito et al. (136) as a polypeptide that interacts with SAPKβ/JNK3 in a yeast two-hybrid screen, and, independently, by Kelkar et al. (155) as an interactor with SAPKγ/JNK1 (Kelkar et al. refer to the protein as JIP3). JSAP1/JIP3, like SAPKβ, is localized selectively in brain and heart (a small amount of JSAP1/JIP3 message is detectable in lung) (136, 155). Two alternative splicing forms of JSAP1/JIP3 may exist: a larger clone (JIP3b) expressed in brain and a smaller clone (JIP3a) found in heart. JSAP1/JIP3 is a large polypeptide (~144 kDa) containing a leucine zipper motif (amino acids 392–427, JIP3a numbering, amino acids 424–459 JIP3b numbering) as well as several coiled-coiled motifs (amino acids 25–155, 265–315, 385–424, 459–515, and 585–615, JIP3b numbering). No other recognizable structural domain features are present (136, 155).

Biochemical studies of JSAP1/JIP3 have yielded conflicting and contradictory results. Both Ito et al. and Kelkar et al. demonstrate that JSAP1/JIP3 can bind all three SAPK isoforms, but binding to SAPKβ is strongest. Binding to other MAPKs (p38 and ERK) is not observed (136, 155). The SAPKβ binding region of JSAP1/JIP3 was mapped to amino acids 115–233 by Ito et al. (136) and refined further to amino acids 202–213 by Kelkar et al. (155). Amino acids 202–213 are strikingly conserved with a segment of the SAPK binding region identified for JIP1 (amino acids 153–164) and suggest a consensus sequence (Arg-X-X-Pro-Thr-Val-Leu-Phe-Pro, where X is any amino acid) for SAPK binding to JIP1 and JSAP1/JIP3 (136, 155). The basic amino acids in this SAPK binding domain are reminiscent of the basic MAPK docking sites present in many MAPK substrates and regulators, that bind to MAPK CD motifs (see sect. II, A). There is no apparent effect on SAPK binding (136, 155).

In contrast, Ito et al. (136) report that prior activation of SAPK in vivo (by expression with truncated, active MEKK1) significantly reduces the binding of SAPK to either unphosphorylated or in vitro phosphorylated JSAP1/JIP3; however, Kelkar et al. (155) report the apparent opposite: prior activation of SAPK by UV radiation increases binding to JSAP1/JIP3 (155). This difference may be due to different modes of SAPK activation (MEKK1 vs. UV radiation), which may enhance or disrupt the binding of the expressed SAPK to scaffold proteins other than JSAP1/JIP3. In any case, activation of the SAPK pathway may regulate differentially the association of SAPK with JSAP1/JIP3.

Consistent with its role in organizing SAPK signaling modules, JSAP1/JIP3 can, like JIP1 and JIP2, associate with upstream elements of SAPK core signaling modules. However, interactions between endogenous JSAP1/JIP3 and endogenous SAPK pathway components have not been assayed, and there is substantial disagreement as to which recombinant, ectopically expressed SAPK pathway components interact with JSAP1/JIP3. Ito et al. (136) observe that in cotransfection/coimmunoprecipitation and in vitro pull down experiments, there is a stable association between JSAP1/JIP3 and SEK1 (but no binding to MKK7 or MKK6). Curiously, Ito et al. (136) also observe that JSAP1/JIP3 can bind MEK1. The significance of MEK1 binding is unclear. Increasing the level of SEK1 expression has no effect on MEK1 binding; likewise, increasing MEK1 expression has no effect on SEK1 binding.
to JSAP1/JIP3, suggesting, but not proving, that the SEK1 binding site is distinct from the MEK1 binding site. The SEK1 binding site has been mapped by Ito et al. to the COOH-terminal region of JSAP1/JIP3 (amino acids 1054–1305, JIP3a numbering) (136). In contrast, Kelkar et al. (155) observed no interaction between JSAP1/JIP3 and SEK1 (or MEK1 for that matter), but observed a strong interaction between JSAP1/JIP3 and MKK7. In both instances JIP3a was used, making it unlikely that these differences are due to the JSAP1/JIP3 splicing isoform used (Fig. 7B).

Ito et al. (136) also observed that JSAP1/JIP3 also binds full-length in vitro translated MEKK1 in in vitro binding assays. In vivo coimmunoprecipitation assays using various MEKK1 truncation mutants indicate that residues 1–640 of MEKK1 are essential for this interaction; however, it is noteworthy that for technical reasons, Ito et al. (136) were unable to detect binding between MEKK1 and JSAP1/JIP3 in vivo. In in vitro assays using [35S]labeled full-length MEKK1, Ito et al. (136) mapped MEKK1 binding to two contact points on JSAP1/JIP3: amino acids 1–305, a weak interaction domain, and amino acids 434–1053, a stronger interaction domain. Ito et al. also observed that JSAP1/JIP3 can weakly interact with a construct consisting of the catalytic COOH-terminal domain of Raf-1 (Raf-C), a MAP3K of the ERK cascade. MEKK1 expression has no competitive effect on Raf-C binding to JSAP1/JIP3, indicating that the binding site for Raf-1 is distal from the MEKK1 binding site. The significance of the Raf-1-JSAP1/JIP3 interaction is unknown (136). It is conceivable that this interaction is indirect, mediated by MEK1. Moreover, binding of full-length Raf-1 to JSAP1/JIP3 has not been observed. In contrast, Kelkar et al. (155) observe no binding between JSAP1/JIP3 and MEKK1, likely due to the use of a truncated MEKK1 construct missing the NH2-terminal 640 amino acids, shown by Ito et al. to be necessary for MEKK1 binding (136, 155). Raf-1 binding to JSAP1/JIP3 was also not observed by Kelkar et al. (155), raising the possibility that the finding of Ito et al. (136), that Raf-1 binds JSAP1/JIP3, is an expression artifact. Instead, Kelkar et al. observe that JSAP1/JIP3 binds MLK3 (but not MLK2 or DLK) (155) (Fig. 7B).

Consistent with their binding results, Ito et al. (136) demonstrate that JSAP1/JIP3 expression significantly enhances the ability of MEKK1 and SEK1 to activate SAPKβ and, to a lesser extent, SAPKα and -γ. Activation by MEKK1 of MEK1 signaling to SAPK is not affected by coexpression with JSAP1/JIP3, in spite of the fact that MEK1 is a weak MEKK1 substrate. The effect of JSAP1/JIP3 on ERK activation by MEKK1/MEK1 was not tested. The turnover of activated SEK1 by JSAP1/JIP3 was also not tested; however, the observation that MEKK1 activation of SAPK reduces SAPK’s affinity for JSAP1/JIP3 suggests that JSAP1/JIP3 organizes signaling complexes containing MEKK1, SEK1, and inactive SAPK, releasing the SAPK once it is activated (136). In a similar vein, Kelkar et al. (155) observed that MLK3 activation of all three SAPK isoforms is strongly enhanced upon coexpression with JSAP1/JIP3.

In cotransfection/coimmunoprecipitation experiments, JSAP1/JIP3 can also homodimerize and heterodimerize with JIP2, but not JIP1 (155). The significance of these interactions is unclear.

The signaling pathways that recruit JSAP1/JIP3-containing signaling modules are not known. However, Kelkar et al. (155) observed that treatment of PC12 cells with NGF significantly induces expression of JIP3 protein coincident with the differentiation of these cells to a neuron-like phenotype. Immunolocalization of JSAP1/JIP3 demonstrates that the protein is localized in axonal growth cones. In contrast, NGF withdrawal or suspension from the substratum, conditions which trigger PC12 cell apoptosis, correlate with caspase-3-mediated cleavage and degradative loss of endogenous JSAP1/JIP3, and JSAP3/JIP1 is a substrate of caspase-3 in vitro. Taken together, these results suggest that conditions supporting PC12 survival and differentiation coincide with JSAP1/JIP3 expression (155). Inasmuch as the SAPKs have been implicated in brain development (see sect. V), the role JSAP1/JIP3 plays in neuronal SAPK regulation remains an important question.

3. The 280-kDa actin binding protein-280 may be a scaffold protein for TNF and lysophosphatidic acid activation of the SAPKs in melanoma cells

The 280-kDa actin binding protein-280 (ABP-280) (also called filamin) is a 280-kDa actin binding protein. Actin binding is mediated by the ABP-280 NH2 terminus. This region is followed by two flexible hinge regions, one two-thirds along its length and the other immediately adjacent to a COOH-terminal homodimerization domain. Structurally, ABP-280 is rod shaped, and aside from the actin binding domain, the various protein interaction domains are distributed among 24 repeats of an ~98-amino acid structure. Notably, repeat 24 itself mediates homodimerization. ABP-280 cross-links actin filaments into orthogonal arrays and contributes to the structure of the cortical actin meshwork, functions attributable to ABP-280’s flexible rod-shaped structure (201). Recent studies indicate that ABP-280 may also serve as a signaling scaffold protein.

In a yeast two-hybrid screen employed to identify novel SEK1 interactors, Marti et al. (196) identified ABP-280 as a SEK1 binding protein. SEK1 binds ABP-280 in vivo and in vitro, and the binding requires a COOH-terminal region of ABP-280 consisting of repeats 21–23C (amino acids 2282–2454) (196). Binding is quite specific, and ERK1, p38α, and MEK1 bind ABP-280 at best very weakly compared with SEK1 binding. However, MEKK1 and
SAPK can coprecipitate with ABP-280 if, and only if, SEK1 is present (196). As discussed below, SEK1 itself has scaffold properties (342) and may permit the formation of MEKK1-SEK1-SAPK-ABP-280 complexes that can be specifically recruited by upstream stimuli (Fig. 7B). Activation of SEK1 with MEKK1 has no effect on the binding of SEK1 to ABP-280; however, ABP-280 appears to be important for the activation of SEK1 and the SAPKs in vivo in melanoma cells, in response to certain specific extracellular stimuli, notably TNF (196).

M2 cells are a human melanoma cell line that has spontaneously lost ABP-280 expression. After plating, these cells show exaggerated and prolonged membrane blebbing, poor spreading, and defective directed cell motility. M2A7 cells are M2 cells wherein ABP-280 has been reconstituted to physiological levels, correcting the defects in motility observed for M2 cells (61, 62). SAPK activation by TNF and lysophosphatidic acid (LPA) is strikingly abrogated in M2 cells, while activation by arsenite, anisomycin, or hyperosmolarity are unaffected. In contrast, activation of SAPK by TNF and LPA is restored in M2A7 cells (196). Thus ABP-280 may serve as a scaffold protein to coordinate the proper activation of the SAPKs by a select subset of activators (TNF and LPA) to the exclusion of others. It is important to note that the loss of TNF activation of SAPK was not observed in other cells wherein ABP-280 expression was ablated. Thus this phenomenon may be cell specific (196).

Recently, it was reported that, in addition to binding SEK1, ABP-280 can bind TNF receptor-associated factor-2 (TRAF2), an adapter protein implicated in TNF and stress activation of the SAPKs (see sect. vE) (179). TRAF2 consists of an NH$_2$-terminal RING effector domain, a central Zn finger domain, and two tandem COOH-terminal TRAF domains (see sect. vE1). ABP-280 binding requires the RING and Zn finger domains of TRAF2. Amino acids 1644–2118 of ABP-280, just upstream of the SEK1 binding site (amino acids 2282–2454), contain the TRAF2 binding domain. Overexpression of ABP-280 in transfected 293 cells abrogates TRAF2 activation of SAPK (and NF-$\kappa$B); however, TRAF2 activation of SAPK is also lost in M2 cells (179). Given that SAPK activation by TNF is lost specifically in M2 cells, and not in other cells (196), the 293 cell result may be attributable to sequestration of endogenous 293 cell SAPK activating machinery from the expressed TRAF2, although this inhibition could be attributable to a prozone effect (see sect. vC1) (Fig. 7B).

4. SEK1 has intrinsic scaffold properties

In addition to its properties as a MEK, SEK1 possesses intrinsic scaffold properties, and as is the case for the yeast MEK Pbs2p and its upstream activators and effectors (242) (Fig. 7B), the intrinsic scaffold properties of SEK1 enable it to form specific, sequential dynamic complexes with both an upstream activator and a substrate. Thus the NH$_2$ terminus (amino acids 1–77) of inactive, dephosphorylated SEK1 can specifically bind one of its upstream activators, MEKK1 (Fig. 7B). A potential splicing isoform of SEK1 wherein the first 34 amino acids have been deleted has not been tested for MEKK1 binding. MEKK1 does not readily interact in vivo or in vitro with MEK-1 or -2, MKK-3 or -6 or with the $\gamma_1$-isoform of MKK7 under conditions in which each MEK is expressed separately with MEKK1. As a consequence, none of these MEKS is as good an in vitro substrate of MEKK1 as is SEK1 (342). However, while MKK7$_{\gamma1}$ does not interact with MEKK1 and is a modest MEKK1 substrate in vitro, several MKK7 isoforms ($\alpha_1$, $\alpha_2$, $\beta_1$, and $\beta_2$) are strongly activated by MEKK1 in vivo (see sect. vF1) (188, 315). These MKK7 isoforms are either NH$_2$ terminally deleted or truncated (315), and their binding to MEKK1 has not been ascertained.

The SEK1-MEKK1 complex can be generated in vitro. Under these conditions, the addition of ATP to the SEK1-MEKK1 complex results in SEK1 activation which, in turn, triggers dissociation of the SEK1-MEKK1 complex (342) (Fig. 7B). Phosphorylated and activated SEK1 then forms a second specific complex, with SAPK, again mediated by the SEK1 NH$_2$-terminal MEKK1 binding domain. This MEKK interaction motif also contains the polybasic MAPK docking site that interacts selectively with the SAPK CD domain (see sect. vE3) (301). Upon phosphorylation and activation of SAPK, this second complex dissociates (342) (Fig. 7B).

D. Regulation of the SAPKs by the GCKs

1. General considerations

GCK is the founding member of a novel family of stress-regulated Ser/Thr protein kinases some of which are selective activators of the SAPKs (163). Accumulating evidence suggests that those GCKs that recruit the SAPKs do so by binding MAP3Ks and elements upstream of MAP3Ks (163, 359). Thirteen mammalian GCKs have been cloned. In addition, there are Drosophila, C. elegans, and Dictyostelium homologs, as well as two S. cerevisiae genes with known phenotypes (163). All GCKs possess NH$_2$-terminal kinase domains that are distantly related to those of the PAKs and Ste20p. The kinase domains are followed by extensive COOH-terminal regulatory domains (CTDs) (163). The distant sequence homology between the kinase domains of the GCKs, Ste20p, and the PAKs led to the initial placement of the GCKs in the Ste20 family. However, the domain arrangement of the GCKs coupled with the fact that these kinases do not possess CRIB motifs and are not directly activated by Rho family GTPases indicate that the GCKs should be considered a distinct protein kinase family (163).
GCKs can be subdivided into two groups based on overall sequence conservation and function: group I GCKs are structurally similar within both the kinase and CTD regions to GCK itself and are specific upstream activators of the SAPKs (163) (Fig. 8A). Group II GCKs are more closely related to the S. cerevisiae GCK Sps1p and are activated in vivo by extreme stresses. Few downstream effectors of group II GCKs have been identified (163). A putative third GCK subgroup consists thus far only of JNK/SAPK inhibitory kinase (JIK) (303).

Seven mammalian group I GCKs have been identified to date: GCK itself, GCK-related (GCKR, also called kinase homologous to Ste20, KHS, Table 4), GCK-like kinase (GLK), hematopoietic progenitor kinase-1 (HPK1), and Nck-interacting kinase [NIK, also called HPK1/GCK-like kinase, HGK; the GCK NIK should not be confused with NF-κB inducing kinase (191), which is also abbreviated NIK], TRAF and Nck interacting kinase (TNK), and NIK-related kinase (Nrk) (Table 4) (73, 96, 131, 145, 151, 159, 277, 294, 319, 354).

There is remarkable structural similarity among the CTDs of group I GCKs. The CTDs of all group I GCKs include two or more proline/glutamic acid/serine/threonine (PEST) motifs and at least two polyproline putative SH3 domain binding sites. Of particular importance, all of the group I kinases possess a highly conserved ~350-amino acid region, the GCK homology domain, at the COOH-terminal end of the CTD. The GCK homology domain can be subdivided into a somewhat hydrophobic, leucine-rich domain and a 142- to 152-amino acid stretch, the COOH-terminal (CT) motif. The leucine residues in the Leu-rich domains are not organized into leucine zippers, nor are these domains sufficiently hydrophobic for membrane insertion. The COOH-terminal-most putative SH3 binding sites of GCK, GCKR, GLK, and HPK1 lie at the NH2-terminal end of the cognate Leu-rich domains. Such a binding site is not evident in the Leu-rich segments of NIK, NTK or Nrk which are closely related to each other, but more divergent from the other mammalian group I GCKs (73, 96, 131, 145, 151, 159, 163, 277, 294, 319, 354).

GCK, GCKR, GLK, and NIK are all activated in vivo by TNF, and TNK may also be a TNF effector (73, 96, 239, 277, 354), and the CT extensions of the CTDs of GCK, GCKR and GLK along with that of HPK1 are quite homologous (Fig. 8A) (73, 131, 151, 159, 277, 319). The CTs of NIK, TNK, and Nrk, while similar to those of the other mammalian group I GCKs, are closer in sequence to each other (96, 145, 294, 354); however, the activation and function of Nrk have not been analyzed (145). Thus structurally, NIK, TNK, and Nrk form one arm of the mammalian group I GCKs while GCK, GCKR, GLK, and HPK1 form a second arm of the mammalian group I GCKs.

Studies of GCK and GCKR indicate that the CT motif is required for binding proteins of the TNF receptor-associated factor (TRAF) family, and, possibly, for gating the binding of MAP3Ks (278, 359) (see sects. IV B3 and IV C). The notion that GCKs can both potently activate the SAPKs as well as stably associate with SAPK core signaling module elements and their upstream activators, in a manner dependent at least in part on the kinase activity of the GCKs (see sects. IV B3 and IV C), suggests that the group I GCKs may coordinately regulate MAPK core signaling modules in conjunction with additional upstream elements.

2. Group I GCKs: selective regulators of the SAPKs

A) GCK. GCK (Table 4) was identified in a subtractive screen for genes preferentially expressed in B-cell germinal centers. Thus, although GCK is present in all tissues examined, its distribution in B lymphocyte follicular tissue is restricted largely to the germinal centers and not the surrounding mantle zones (151). Germinal centers are sites of B-cell maturation (notably Ig class switching) and selection, processes that are driven by ligands of the TNF family (CD40L, CD30L, TNF, in particular) (34, 200). For example, mice in which the gene for the type 1 TNF...
receptor has been disrupted display little or no germinal center formation (200). Accordingly, it was logical to propose that GCK, which is selectively expressed in the germinal center, might be an effector for TNF family ligands and might signal to the SAPKs. Indeed, endogenous GCK, upon immunoprecipitation from several B cell lines, is significantly activated in vivo by TNF (239). Moreover, transient expression of GCK results in potent SAPK activation in the absence of external stimulus. In contrast, expression of GCK does not result in activation of the ERKs, p38s, or NF-kB (239, 277). Thus, like MEKK1 and the MLKs (see sects. ivG2 and ivG3), GCK is a specific SAPK activator.

Although capable of undergoing activation in vivo by upstream stimuli, both endogenous and recombinant GCK, like many stress-activated MAP3Ks, exhibits substantial basal activity in vitro assays, or upon expression in vivo, unless expressed at extremely low levels (151, 239, 359). This finding suggests that GCK is activated in vivo either by limiting concentrations of an inhibitor lost during immunoprecipitation or stoichiometrically titrated out upon GCK overexpression. Alternatively, GCK might be regulated by oligomerization, a process that could be mimicked by immunoprecipitation and/or overexpression. In either case, the CTD is the region of the GCK polypeptide likeliest to mediate GCK regulation and substrate recognition (163). In support of this idea, transient expression of the free GCK-CTD, or a GCK construct (Lys44Met) that is completely devoid of kinase activity results in SAPK activation (239, 359). As discussed in sections ivB3 and ivC, the GCK-CTD binds both GCK regulators and effectors.

b) GCKR. Using a database mining strategy, Shi and Kehrl (277) identified GCKR (also called kinase homologous to STE20, KHS, Table 4) as a protein kinase highly similar to GCK. Independently, Tung and Blenis (319) cloned GCKR as part of a screen to identify novel mammalian homologs of STE20. GCKR is ~60% identical to GCK throughout its primary sequence. Most notably, the GCK homology domain (Leu-rich and CT motifs) of the CTD is highly conserved (151, 163, 277, 319). Like GCK, GCKR is a selective activator of the SAPKs. The ERKs, p38s and NF-kB are not activated upon expression of GCKR (277, 319). For reasons that are unclear, both endogenous and recombinant GCKR possess considerably lower basal activity than GCK both in vivo and in vitro, and in contrast to GCK itself, transient expression of the GCKR-CTD does not result in significant SAPK activation (277, 319).

Endogenous GCKR is activated in vivo by TNF, CD40, and UV radiation (45, 277, 278). UV activation of GCKR is likely mediated by UV-induced clustering of TNF receptors (256). It is likely that GCKR is an important effector for TNF, at least in some cell types. In support of this, antisense constructs of GCKR, when expressed in 293 cells, can completely inhibit TNF activation of the SAPKs (277) (see sect. ivC).

c) GLK. GLK (Table 4) is a third type I GCK homolog that is, like GCK and GCKR, a selective activator of the SAPKs. The CTD of GLK is strikingly conserved with those of GCK and GCKR, especially within the GCK homology domain. Consistent with this close similarity, endogenous GLK is activated in vivo by TNF (73).

d) HPK1. HPK1 (Table 4) was identified in a subtractive screen for genes preferentially expressed in cells of the hematopoietic lineage. HPK1 shares many of the structural features of group I GCKs, although it is more distantly related to GCK than is GCKR and GLK. HPK1 has four consensus SH3 domain binding sites (131, 159, 163). The COOH-terminal most SH3 binding site interacts with the SH3 domain of the mixed lineage kinase (see sect. ivG3) MLK3 (159) (discussed in sect. ivG1). Transient overexpression of HPK1 results in vigorous activation of the SAPKs. p38 is activated only very weakly, and no ERK activation is observed upon expression of HPK1 (131, 159, 163).

Two recent studies indicate that HPK1 may be regulated by SH2/SH3 adapter proteins that couple to receptor and/or nonreceptor Tyr kinases. Thus the NH2-terminal two SH3 binding sites of HPK1 interact with the COOH-terminal SH3 domain of SH2/SH3 adapter protein Grb2 (5), which is also a key regulator of Ras (195). The Grb2-HPK1 interaction is not affected by extracellular stimuli; instead, treatment of cells coexpressing Grb2 and HPK1 results in a translocation of the Grb2-HPK1 complex to the membrane. The significance of the HPK1-Grb2 interaction is still somewhat unclear, however, given that coexpression with Grb2 has no effect on HPK1 activity (5).

In contrast, Crk and CrkL, two closely related SH2/SH3 adapter proteins, can bind to the second and fourth polyproline SH3 binding sites of HPK1. As with the Grb2-HPK1 interaction, the Crk(CrkL)-HPK1 interaction enables the EGF-dependent recruitment of HPK1 to the EGF receptor (181). Support for the idea that the Crk(CrkL)-HPK1 interaction is physiologically relevant comes from the observation that coexpression of Crk or CrkL with HPK1 results in HPK1 activation and synergistic SAPK activity (181). Although the SAPKs are usually only modestly activated by mitogens such as EGF that signal through receptor Tyr kinases, the Grb2-HPK1 and Crk/ CrkL-HPK1 interaction could explain in part the regulation of the SAPKs by any of several nonreceptor Tyr kinases such as Btk and Pyk2 that strongly activate the SAPKs in vivo (see sect. ivB) (Figs. 6B and 8B).

In this regard, it is noteworthy that Btk and Pyk2 are putative effectors for trimeric G protein-coupled receptors (75, 141). Several MAP3Ks upstream of the SAPKs [the SAPK-specific MEKK1 (see sect. ivB1) and MLK3 (see sect. ivG2) for example] bind Rac1 in a GTP-dependent
manner. Rac1 has also been implicated in trimeric G protein signaling to SAPK; and C3G, a GEF for Rho family GTPases, is, like HPK1, recruited by Crk/CrkL (33). Accordingly, it is possible that one function of HPK1 translocation is to colocalize it and, possibly, other MAP3K activators, with effector MAP3Ks simultaneously translocated to the plasma membrane perhaps via binding to Ras superfamily GTPases (163).

E) NIK. Murine NIK (also called HPK1/GCK-like kinase, HGK, Table 4) was cloned as part of a two-hybrid screen to identify novel polypeptides that interact with the SH2/SH3 domain-containing adapter protein Nck. A human homolog was cloned by degenerate PCR using primers based on sequences conserved in the GCK family (294, 354). Of the mammalian group I GCKs, NIK, and its close relatives TNIK (below) and Nrk, are the most distinctly related. Still, functionally speaking, NIK shares many of the same features of the group I GCKs. Notably, NIK transient overexpression strongly and specifically activates the SAPKs (294, 354). Moreover, as with GCK, expression of the free NIK CTD significantly activates the SAPKs, albeit to a lesser degree than does wild-type NIK (294).

NIK contains two consensus SH3 binding motifs (amino acids 574–579 and amino acids 611–616), both of which can interact with the SH3 domains of Nck (294). Nck is thought to be involved in the regulation of the actin cytoskeleton, and one function of NIK may be to mediate changes in cell motility and shape. Indeed, a Drosophila ortholog of NIK, Misshapen, is required for embryonic dorsal closure (see sect. ivC) (295). NIK can be activated by TNF (354), and recent studies of Misshapen signaling indicate that it (and, by extension, NIK), like GCK and GCKR, is an effector for TNF receptor-associated factors (TRAFs) (163, 183, 278, 359) (see sect. ivC).

As with the Crk/CrkL-mediated activation of HPK1 by EGF (181), the interaction between NIK and Nck couples NIK to Eph family Tyr kinase receptors. The EphRs are critical to embryonic patterning in the nervous and vascular systems. NIK is substantially activated by several Eph family Tyr kinases, notably EphB1R and EphB2R (14). Stimulation of EphB1R leads to the formation of a complex consisting of NIK, Nck, and p62\textit{dok} (14). p62\textit{dok} is a PH-domain-containing adapter that is Tyr phosphorylated by activated EphRs and other Tyr kinases. Upon Tyr phosphorylation, p62\textit{dok} recruits SH2 domain-containing effectors (355) and RasGAP (201, 202). Within the NIK-Nck-p62\textit{dok} complex, NIK is associated with the SH3 domains of Nck, and the SH2 domain of Nck itself is associated with Tyr phosphorylated p62\textit{dok}. Studies with dominant inhibitory NIK constructs indicate that NIK is critical to several downstream consequences of EphB1R including SAPK activation and activation of integrins (14) (Fig. 8B).

F) TNIK. TRAF2 and Nck-interacting kinase (TNIK) is quite similar in structure overall to NIK (~90% identity at the amino acid level within the kinase domain, amino acids 1–306, ~90% within the Leu-rich and CT motifs, amino acids 1019–1360, and ~53% within the remainder of the CTD, amino acids 307–1018). As with NIK, TNIK expression results in potent and specific SAPK activation. In contrast to NIK and GCK, however, the TNIK CTD is equally potent at SAPK activation as is full-length TNIK (96). TNIK’s nonconserved intermediate region, amino acids 307–1018, contains both the binding site for TRAFs and Nck (below). In addition to full-length TNIK (TNIK1), at least seven hnRNA splicing isoforms of TNIK may exist. These splicing variations are all deletions within the intermediate region of the TNIK CTD, upstream of the Leu-rich/CT motif (TNIK2, Δ447–475; TNIK3, Δ537–591; TNIK4, Δ795–802; TNIK5, Δ447–475/537–591; TNIK6, Δ447–475/795–802; TNIK7, Δ537–591/795–802; and TNIK8, Δ447–475/537–591/795–802) (96). TNIK can interact with Nck in vivo, although the role of this interaction is unclear. Several of the splicing deletions disrupt one potential SH3 binding site at amino acids 590–593. However, it has not been determined which potential SH3 binding site (amino acids 590–593, which corresponds to the SH3 binding site on NIK at amino acids 574–579, or a second potential site at amino acids 669–673, which corresponds to the SH3 binding site on NIK at amino acids 611–616) is necessary for Nck binding. TNIK can also interact with TRAF2, although there is no evidence that TNIK is regulated by TNF family agonists. The TRAF binding region has been localized to the intermediate region upstream of the Leu-rich motif of the CTD. The impact of TNIK mRNA splicing on TRAF binding is unknown (96). It is tempting to speculate that the multiplicity of TNIK isoforms permits multiple forms of interactions with upstream activators, or the generation of endogenous, inhibitory TNIK species, missing specific binding sites, which serve to sequester TNIK from its effectors.

3. Group II GCKs: activation by extreme stresses

Mammalian group II GCKs include Ste20-like oxidant stress-activated kinase-1 (SOK1), kinase responsive to stress (Krs)-1, mammalian sterile twenty-like-1 (MST1)/Krs2, MST2, MST3, and lymphocyte-oriented kinase (LOK) (55, 57, 162, 238, 269, 304). These enzymes are structurally more similar to S. cerevisiae Sps1p than to group I kinases. Group II GCKs are less well understood, and, with the exception of MST1/Krs2 (109), apparently do not activate any of the known MAPK pathways. Although group II GCKs share substantial catalytic domain homology with group I GCKs, their CTDs differ significantly from those of the group I enzymes.

Like group I GCKs, mammalian 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 (55, 57, 162, 238, 269, 304). However, Krs1, MST1/Krs2, and SOK1 can be activated substantially in vivo by different environmental stresses. Krs1 and MST1/Krs2 are activated by extreme heat shock, high concentrations of arsenite, the general protein kinase inhibitor staurosporine, and the phosphatase 2A inhibitor okadaic acid (56, 304). MST1/Krs2 can also be activated in vitro by phosphatase 2A (56). Thus these two group II GCKs may be activated by contemporaneous phosphorylation and dephosphorylation. MST1/Krs2 is also activated in vivo upon engagement of the proapoptotic receptor Fas (109). SOK1, as its name implies, is strongly activated by oxidative stress (238). SOK1 is also activated by ischemic injury and depletion of the cellular ATP pool (240). In all cases, SOK1 activation appears to require the generation of reactive oxygen intermediates as well as elevated levels of cytosolic free Ca\(^{2+}\) (240). Stimuli that recruit LOK and MST3 are unknown (162, 269).

Certain of the group II GCKs display enzymologic properties that may yield clues as to the mechanisms of regulation of kinases of the entire GCK family. Thus SOK1 and MST3 autoactivate upon autophosphorylation in vitro (238, 269), and SOK1 and MST1/Krs2 spontaneously homodimerize in vivo (56, 238), suggesting that oligomerization and autophosphorylation may play a role in GCK family kinase activation.

MST1/Krs2 is the only group II GCK for which potential downstream targets have been identified. MST1/Krs2 is activated by caspase-3-mediated cleavage at a consensus Asp323-Glu-Val-Asp-Ser caspase cleavage site that neatly bisects the enzyme into a free, active catalytic domain moiety and a free regulatory domain (109). MST1/Krs2 cleavage occurs in response to two of its known upstream activators: staurosporine and the phosphatase 2A inhibitor okadaic acid (56, 304). MST1/Krs2 can also be activated in vitro by phosphatase 2A (56). Thus these two group II GCKs may be activated by contemporaneous phosphorylation and dephosphorylation. MST1/Krs2 is also activated in vivo upon engagement of the proapoptotic receptor Fas (109). SOK1, as its name implies, is strongly activated by oxidative stress (238). SOK1 is also activated by ischemic injury and depletion of the cellular ATP pool (240). In all cases, SOK1 activation appears to require the generation of reactive oxygen intermediates as well as elevated levels of cytosolic free Ca\(^{2+}\) (240). Stimuli that recruit LOK and MST3 are unknown (162, 269).

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4. JIK, an inhibitor of mitogen-activation of SAPK: a third class of GCK

JIK was recently identified as a novel member of the GCK family. The sequence of JIK differs significantly from group I and II GCKs, and, given its apparently novel function, JIK may represent a third GCK subgroup. JIK was cloned in a two-hybrid screen for interactors with Eps8, an SH3 domain-containing protein identified as a substrate for the EGF receptor (303). Nevertheless, stimulation of cells with EGF does not result in the translocation of JIK to the EGFR complex. Remarkably, however, JIK Ser/Thr kinase activity is almost completely inhibited upon treatment of cells with EGF. Coincident with this, transient overexpression of JIK blocks the modest (2-fold) activation of SAPK incurred by EGF, but not the more robust SAPK activation stimulated by UV light or anisomycin (303). On the basis of these results, it is conceivable to speculate that in vivo, a component of EGF regulation of SAPK involves relieving an inhibition mediated by JIK. JIK has no effect, positive or negative, on ERK, p38, or ERK5 activity (303).

E. Regulation of the SAPKs and p38s by Adapter Proteins That Couple to TNF Family Receptors

Upon binding ligand, receptors of the TNF family (the TNFR family) can elicit a wide variety of inflammatory responses and are critical to immune cell development, innate and acquired immunity, as well as the pathogenesis of a number of diseases such as arthritis, septic shock, and, possibly, type 2 diabetes mellitus. Accordingly, this family of receptors is among the most important activators of the SAPKs, p38s. The TNFR superfamily includes TNFR1, TNFR2, the lymphotoxin-β (LT-β) receptors, CD40, CD27, Fas, receptor activator of NF-κB (RANK, also called osteoprotegerin, OPG, or TNF-related activation-induced cytokine, TRANCE, receptor), the death receptors (DRs)-3–5, the TNF-related apoptosis inducing ligand receptors (TRAILRs 1–4, TRAILR2 is the same as DR5), CD30, OX40, 4–1BB, and the p75 neurotrophin receptor (p75NTR). The receptors for IL-1 and the Toll-like receptors (TLRs, which, among other things, include receptors for endotoxin) are structurally completely divergent from the TNFR family; however, their mode of signaling is quite similar to TNFRs and, accordingly, these receptors are often grouped with the TNFR superfamily. Several viral genes also encode TNFR family polypeptides; the latent membrane protein-1 (LMP1) of Epstein-Barr virus is an example. With the exception of the IL-1R and the TLRs, most TNFR superfamily receptors share a modest structural similarity within their extracellular domains, in keeping with the structural conservation of many TNF superfamily ligands. In contrast, despite the similarities in TNF superfamily signaling programs and
modes of receptor activation, the intracellular extensions of TNFR family receptors are quite divergent (7, 17, 18, 284, 316, 323).

1. The protein recruitment model for TNFR family signaling

Receptors of the TNFR family possess no intrinsic enzymatic activity. Instead, upon binding ligand, these receptors homotrimmerize or heteroligomerize with receptor accessory proteins. Receptor oligomerization is thought to trigger conformational changes that initiate signal transduction. The protein recruitment hypothesis poses that receptor oligomerization enables the binding of signal transducing polypeptides that recruit downstream targets (322, 323).

A) DEATH DOMAINS AND SIGNALING. Several TNFR family receptors (TNFR1, Fas, TRAILR and the DRs, in particular) contain an 82- to 102-amino acid extension, the death domain (301–304). Death domains mediate homotypic and heterotypic protein-protein interactions and are critical for nucleating receptor-effector complexes and implementing several signaling programs including apoptosis (322, 323). The type-1 TNFR (TNFR1) can recruit all of the known signaling pathways activated by TNF. Signal transduction by TNFR1 is now understood in considerable detail. Upon ligand-induced TNFR1 trimerization, the TNFR1 death domain binds the death domain of the platform adapter protein TNFR-associated death domain protein (TRADD) (130, 322, 323). In contrast, TNFR2 does not possess a death domain, and, upon trimerization, instead binds directly to TNFR-associated factor (TRAF) proteins through a TRAF domain binding site (below) (257).

TRADD consists of a COOH-terminal death domain and an NH2-terminal domain, the TRAF interaction domain, that binds TRAF proteins (see below). TRADD is a critical proximal component in the recruitment by TNFR1 of downstream targets. Consistent with this, overexpression of TRADD can activate many TNF signaling pathways including apoptosis and NF-κB (130). In contrast, although TRADD can recruit TRAF2 and receptor interacting protein (RIP) (129, 128), two key upstream activators of the SAPKs and p38s (185, 223, 253, 359) (see sect. wE2), TRADD overexpression does not activate the SAPKs (185, 223, 253). The reason for this is unknown. The interaction between the TRADD death domain and that of TNFR1 is thought to trigger the binding of additional death domain proteins to the TRADD death domain. An example is Fas-associated death domain protein (FADD), an adapter protein that couples TNFR1 to the apoptotic machinery. FADD associates with TRADD through a homotypic interaction between the two proteins’ death domains (129). TRAF proteins are also recruited to the TRADD-TNFR1 complex through an interaction between the TRAF interaction domain of TRADD and the TRAF domains of TRAF2 (below) (7, 129, 322, 323) (Fig. 9A).

B) TRAFs. The TRAFs are an emerging family of proinflammatory signal-transducing adapter proteins that are important for the activation of a number of pathways in response to TNF family ligands (7, 323). Six mammalian TRAF polypeptides (TRAFs 1–6) have been identified. The TRAFs consist of two tandem COOH-terminal TRAF domains (TRAF-N followed by TRAF-C), a central zinc finger motif and, with the exception of TRAF1, an NH2-terminal RING finger motif (7, 323). The TRAF domains are responsible for binding upstream activators such as TRADD, as well as some TRAF effectors. The function of the Zn finger domains is unclear; however, the RING fingers are important for the activation of downstream effectors (7, 13, 96, 185, 182, 223, 253, 278, 299, 323, 359) (Fig. 9).

Details of the structural features required for TRAF binding to upstream proteins are emerging. TRAF-1 and -2 were purified and cloned based on their interactions with the intracellular extension of TNFR2, with which both proteins interact directly (7, 257, 323). Since then, the repertoire of polypeptides that bind TRAFs has expanded considerably, and a greater understanding has developed of the structural basis of TRAF proteins binding to their activators/effectors. Crystallographic studies have shown that the TRAF domains of TRAF2 trimerize upon interaction with trimerized upstream activating receptors (204, 231). In these arrangements, TRAF domains bind to one of two consensus sequences: Pro/Ser/Ala/Thr-X-Gln/Glu-Glu, the predominant site, or a secondary sequence, Pro-X-Gln-X-X-Asp, where X is any amino acid. These sites are present in TNFR family receptors and some TRAF2 binding proteins (356).

A notable exception to this rule is TRADD, which couples TNFR1 to TRAF2 (130, 129). TRADD contains neither consensus TRAF2 TRAF domain binding site (130, 356). Thus other TRAF domain binding motifs likely exist, and these other sites may link TRADD to TRAF2. This leaves open the possibility that, once TRAF2 is bound to TRADD, the regions of the TRAF2 TRAF domains that interact with the consensus TRAF2 binding motifs are free to interact with other proteins. Conversely, TRAF2 associated with a “consensus” TRAF2 binding site on one of its binding partners may have additional sites on its TRAF domains free to associate with other proteins (Fig. 9B). Thus TRAF domains could conceivably associate contemporaneously with upstream and downstream polypeptides each with distinct TRAF domain binding motifs.

C) RECEPTOR INTERACTING PROTEIN. Receptor interacting protein (RIP) was cloned as a polypeptide that could interact in a two-hybrid screen with the death domain-containing receptor Fas. Indeed, ectopic expression of RIP results in rapid apoptosis and, in parallel, activation of NF-κB (287, 310). Subsequent studies using cell lines in
which expression of the endogenous RIP gene is ablated have shown that RIP is not involved in Fas proapoptotic signaling but is instead critical TNF activation of NF-κB, a contention supported by subsequent gene disruption studies (156, 310). RIP consists of a COOH-terminal death domain, an NH2-terminal Ser/Thr kinase domain, and an intermediate domain that likely forms a coiled-coil structure (287).
RIP does not interact directly with TNFR1. Instead, the RIP death domain binds to that of TRADD in a TNF-dependent manner (128). The RIP kinase and intermediate domains can also bind to the TRAF domains of TRAF2 (299), and TNF treatment is thought to result in the formation of a TRADD-RIP-TRAF2 complex at the membrane (Fig. 9A).

2. TRAF-2, -5, -6, and RIP can activate the SAPKs and p38s

Inasmuch as TRAFs and RIP appear to be constitutively active upon overexpression, nonspecific artifacts of overexpression could lead to incorrect inferences concerning function. Still, in spite of this, overexpression studies have provided valuable insight into the possible mechanisms by which the TRAFs and RIP act.

Transient overexpression of TRAF2 results in potent SAPK and p38 activation (185, 223, 253, 359). The RING domain appears necessary for relaying signals from TRAFs to downstream effectors, and deletion of the TRAF2 RING finger domain abrogates completely the ability of TRAF2 to recruit the SAPKs and p38s. Moreover, ∆RING-TRAF2 can act as a dominant inhibitor of TNF activation of the SAPKs and p38s (185, 223, 253, 359). Targeted disruption of traf2, or transgenic expression of ∆RING-TRAF2 abolishes TNF activation of SAPK; however, IL-1 activation of SAPK is unaffected (p38 was not tested) (176, 357). Thus TRAF2 is a key rate-limiting signal transducer for coupling TNFRs to the SAPKs and, possibly, the p38s. TRAF-5 and -6, when expressed ectopically, can also strongly activate the SAPKs; however, overexpression of TRAF-1, -3, and -4 does not result in SAPK activation (286). Inasmuch as TRAF2 appears to be the dominant TRAF coupling the TNFRs to the SAPKs, activation of the SAPKs by TRAF-5 and -6 may couple the SAPKs to other signaling pathways.

RIP, like TRAF2, is, when overexpressed, a potent activator of both SAPK and p38. The RIP intermediate domain is both necessary and sufficient for this function (185, 359). Expression of a RIP construct wherein the intermediate domain is deleted (RIP-ΔID) effectively inhibits TNF activation of both SAPK and p38 (359). Moreover, RIP and TRAF2 can interact directly in vivo (299), raising the possibility that TRAF2 and RIP act collaboratively to recruit the SAPKs. However, gene disruption studies indicate that deletion of rip does not affect TNF activation of SAPK but, instead, prevents activation of NF-κB (156, 310), suggesting that RIP is not rate limiting for SAPK activation by TNF, an idea supported by the observation that several elements upstream of the SAPKs, including GCK, GCKR, ASK1, and MEKK1 also bind and are activated by TRAFs independently of RIP (see sect. iv, B3, C, and D). A similar situation may exist for p38.

3. TRAF2 also couples ER stresses to the SAPKs

A recent study has implicated TRAFs in the coupling of endoplasmic stress (ER stress) signaling to the SAPKs (321). Stress in the ER is induced by environmental perturbations such as oxidants, heat, ionizing radiation, or chemical denaturants such as tunicamycin (152). These stresses lead to the accumulation of misfolded proteins in the ER lumen. Misfolded proteins are sensed by a novel family of enzymes, the Ire1s, mammalian homologs of S. cerevisiae Ire1, the product of the inositol auxotrophy gene ire1 (also called ernl) (152, 215, 274, 311, 328). Ire1p possesses both endoribonuclease and protein Ser/Thr kinase activity, each of which is required for biological function (152, 311).

In mammalian cells, the ER stress response can be triggered by tunicamycin (152), and tunicamycin leads to the robust activation of the SAPKs (166). Tunicamycin and other ER stresses lead to the oligomerization and activation of mammalian Ire1p (311, 328), which in turn activates SAPK. Moreover, overexpression of mammalian Ire1α is sufficient to activate SAPK, and ER stress activation of SAPK is ablated in ire1α-/- cells and is blocked by kinase-dead Ire1p (321).

Urano et al. (321) performed a yeast two-hybrid screen using the intracellular extension of Ire1p as bait and, surprisingly, isolated TRAF2. Consistent with the possibility that TRAF2 is a genuine effector for Ire1, ER stress leads to the association of endogenous TRAF2 and endogenous Ire1p, and dominant inhibitory TRAF2 can block activation of SAPK by overexpressed Ire1p (321). Inasmuch as Ire1p activation involves homologimerization, it is likely that this triggers the binding and oligomerization-dependent activation of TRAF2 (152, 274, 321). Thus TRAFs may serve in a broad range of signaling pathways, transducing not only cytokine signals but ER stress signals as well (Figs. 8 and 9A).

4. IL-1 and TLR signaling pathways also follow the protein recruitment model and employ TRAF6

IL-1 likely requires TRAF6 to signal to the SAPKs, and targeted disruption of traf6 severely blunts IL-1 activation of the SAPKs and NF-κB (186). IL-1 signaling, like that of TNFRI, follows the protein recruitment paradigm (Fig. 9A). IL-1 binding to its receptor (IL-1R) triggers heterodimerization of the IL-1R with the IL-1R accessory protein (IL-1RacP), a transmembrane protein necessary for IL-1 signal transduction. The IL-1R-IL-1RacP heterodimer then recruits the death domain adapter protein MyD88 (333).

In a manner similar to RIP recruitment by TRADD, the death domain of receptor-bound MyD88 binds Ser/Thr
kinases of the IL-1R-associated kinase (IRAK) family. IRAKs (IRAK-1 and -2) consist of a short NH2-terminal extension required for binding to the MyD88 death domain, a central kinase domain, and a COOH-terminal extension necessary for recruiting downstream target proteins (29, 221).

As with TRAF6, ectopic expression of IRAKs activates NF-κB and the SAPKs, and disruption of irak1 substantially diminishes IL-1-stimulated activation of NF-κB (13, 146, 307). Upon recruitment to the IL-1R complex, IRAK1 binds TRAF6 in a reaction involving the TRAF6 TRAF domains and the COOH-terminal effector region of IRAK1, forming a MyD88-IRAK1-TRAF6 complex analogous to the TNFR-associated complex of TRADD, RIP, and TRAF2 (Fig. 9) (7, 333). The functional interrelationship between TRAF6 and IRAK1 in SAPK and NF-κB activation is unclear.

Structurally, IRAKs are orthologs of Drosophila pelle, which encodes a Ser/Thr kinase recruited by Toll, a Drosophila IL-1-like signaling pathway involved in innate immunity and in the establishment of dorsoventral polarity (135). Toll itself shares significant structural similarity within its intracellular extension with IL-1R, in spite of the fact that the extracellular extensions of these receptors are quite divergent (6, 135).

Mammalian toll-like receptors (TLRs) have been isolated that share an even greater overall similarity with toll (6). These receptors are central to the acute phase response and to the pathogenesis of septic shock, a major cause of mortality among hospitalized patients. Both the TLRs and toll possess extracellular leucine-rich repeats, as well as intracellular extensions homologous to that of IL-1R (6). Consistent with this structural similarity, TLRs and toll likely serve to recognize a subset of structurally conserved molecules present on the surfaces of bacterial pathogens; binding of these bacterial proteins initiates the acute phase response (6, 17, 17). Thus genetic complementation studies recently revealed that TLR4 is the main component of a receptor complex for the lipid A component of endotoxin, a major causative agent for septic shock (17, 18, 177, 235). Initiation of TLR signaling requires the formation of a multiprotein complex analogous to the IL-1R-IL-1RAcP complex. Thus lipid A is thought to interact with and trigger the formation of a functional complex that consists of TLR4, the cell surface protein CD14 and a third polypeptide, monocyte-derived-2 (MD-2) (17, 18, 177, 235, 280). Activated TLR receptor complexes then recruit intracellular effector proteins. Thus TLR2 is thought to signal through TRAF6 and IRAK1, thereby recruiting NF-κB and AP-1, and prompting the release of TNF and IL-1 to initiate the acute phase response (350).

5. Daxx may couple FasL to the SAPKs: conflicting findings

Fas is a widely expressed cell death receptor that, among other things, is crucial to immune cell regulation where it governs in part the apoptotic elimination of autoreactive T lymphocytes. Engagement of Fas by FasL triggers apoptosis through a process that involves the adapter protein Fas-associated death domain protein (FADD). The death domain of FADD interacts with that of activated Fas to signal apoptosis through activation of the caspase family of proapoptotic cysteine proteases (284, 323, 358).

Daxx is a novel protein that was isolated in a two-hybrid screen for Fas interactors. On the basis of this interaction, it was proposed that Daxx served as an adapter, coupling Fas to the SAPKs (352). Daxx overexpression can also trigger apoptosis, suggesting a FADD-independent apoptotic pathway emanating from Fas. The SAPKs appeared to be required for Daxx-induced cell death but only in a subset of the cell lines tested. Thus Daxx-induced apoptosis was blocked by kinase-inactive SEK1 in 293 and L929 cells, but not in HeLa cells (352). Daxx does not possess a death domain. Instead, a COOH-terminal death domain interaction motif (amino acids 625–739) of Daxx may bind directly to the Fas death (see below) domain, but not to the death domain of FADD. Expression of a construct consisting solely of the Fas binding region of Daxx was shown to block both Fas-induced SAPK activation and apoptosis (352). As discussed in section II, Daxx may recruit the SAPKs by directly binding and activating ASK1 (36).

Whereas these original studies implicated Daxx in Fas-induced cell death and SAPK activation, more recent studies call these findings into question. Thus Fas mutants wherein the death domain fails to interact with FADD are completely unable to trigger apoptosis in response to FasL, despite the fact that Daxx binding and Fas-induced SAPK activation by these mutant receptors are unimpaired (37). More importantly, targeted disruption of daxx is embryonic lethal, with lethality occurring as a result of massive cellular apoptosis (208). Taken together, these results argue in favor of an antiapoptotic role for Daxx, rather than a proapoptotic role. Finally, the exact role of Daxx as an effector for Fas is also unclear. Several laboratories have cloned Daxx independently of its association with Fas; and in some instances, Daxx has been isolated in yeast two-hybrid screens as an interactor with nuclear proteins such as DNA methyltransferase. Paradoxically, specific antisera were used in one study to demonstrate a predominantly nuclear localization for Daxx, and in another Fas-dependent association of endogenous Daxx with endogenous, cytosolically localized ASK1 (37, 208).

Studies by Reed and co-workers (313) further sup-
port the idea that Daxx is a nuclear protein. These studies indicate that Daxx associates with PML oncoenic domains (PODs), regions of chromatin that bind PML proteins, a class of oncoenic transcriptional regulators. Daxx association with PODs correlates with transcriptional repression and enhancement of Fas apoptosis that is apparently completely independent of any regulation of the SAPKs (313). In contrast to the original characterization of Daxx as a Fas binding SAPK activator, Reed and co-workers (313) observed that Daxx could not associate with Fas and did not, upon transient overexpression, activate the SAPKs. The reasons for these discrepancies are unclear and might merely reflect trivial issues such as transfection levels, in situ masking of epitopes to which anti Daxx antisera are directed, or the cell types in which the experiments were performed. Clearly, however, more work is needed before the role of Daxx in signaling to the SAPKs is established.

IV. MOLECULAR MECHANISMS COUPLING STRESS-ACTIVATED MAP3Ks TO UPSTREAM SIGNALS

A. General Considerations

Section II outlined the basic elements of known mammalian stress-activated MAP3K →MEK →MAPK core modules, while section III described upstream components thought to couple to these core pathways. Still, despite considerable progress in the identification of SAPK, and p38-activating MAP3Ks, and the identification of proximal signaling components, such as Ras superfamily and trimeric GTPases, GCKs and TRAFs, that are thought to couple to SAPK and p38 core pathways, the molecular mechanisms by which stress-activated MAP3Ks are regulated by events at the cell membrane remain obscure. What little is known suggests that stress-activated MAP3Ks either 1) bind directly to upstream activating regulatory proteins or 2) couple to other protein kinases that, in turn, bind upstream regulatory molecules, and 3) activation of these MAP3Ks by upstream components involves in part regulated oligomerization.

B. MEKK1 May Lie Downstream of Ras and Rho Family GTPases, GCK Family Kinases, and TRAFs and May Be Targeted to an Apoptotic Pathway Upon Caspase Cleavage

Three mechanisms for regulation of MEKK1 are suggested by existing data: 1) recruitment by Ras and Rho family GTPases, 2) site-specific cleavage to generate an active fragment that triggers apoptosis, and 3) binding to TRAFs and GCK family kinases that, in turn, couple to complexes associated with TNFR family receptors.

1. Ras superfamily GTPases and elements coupled to receptor and nonreceptor Tyr kinases

MEKK1 can interact with Ras, Rac1, and Cdc42Hs in a GTP-dependent manner (89, 260). Moreover, kinase-dead forms of the MEKK1 catalytic domain (Δ1–1221/ K1253M) can block activation of SAPK by Rac1 and Cdc42Hs (89). MEKK1 does not contain a consensus CRIB motif, and deletion of the entire regulatory domain does not affect Rac1 or Cdc42Hs binding; the interaction of MEKK1 and Ras or Rho family GTPases likely requires the MEKK1 kinase domain (89, 260).

In apparent contrast to the activation of Raf-1 upon coexpression with active Ras, coexpression of Ras superfamily GTPases with MEKK1 has no detectable effect on MEKK1 activity, largely due to MEKK1’s high degree of basal activity, and the functional significance of the GTPase-MEKK1 interaction is unclear. Indeed, MEKK1 and SEK1 form a tight complex in vivo (342) (see sect. IVc4), and, accordingly, inhibition of G protein signaling by K1253M MEKK1 could merely reflect sequestration of SEK1. It is possible that one function of GTP-dependent Ras, Rac, or Cdc42 binding to MEKK1 is to translocate MEKK1 to sites of activation by other polypeptides or second messengers, or to sites where substrates are located.

The GTP-dependent coupling of MEKK1 to Ras superfamily GTPases indicates that MEKK1 may be an effector for those agonists that recruit the SAPKs through Ras, Rac1, or Cdc42Hs-dependent mechanisms. These include agonists coupled to Tyr kinases such as mitogens and agents that signal through heterotrimeric G proteins (195). In this regard, it is noteworthy that endogenous or recombinant MEKK1 can interact with the SH2/SH3 adapter protein Grb2 (241). This interaction requires the COOH-terminal SH3 domain of Grb2 and amino acids 233–531 of MEKK1 (amino acids 233–291) which includes the COOH terminal of two SH3 binding sites (see sect. IVG2) (241, 344). EGF recruits the Grb2-MEKK1 complex to the Tyr-phosphorylated form of the SH2 adapter protein Shc, already bound to the Tyr phosphorylated EGFR (241). EGF also recruits to the membrane complexes of either Grb2 or Crk/CrkL and the GCK family kinase HPK1 (a putative regulator of MEKK1, see sect. IVc2 and Refs. 131, 159) as well as the Ras-activating Grb2-mSOS complex to the EGFR (5, 181, 195); and Eph Tyr kinases recruit and activate NIK/Nck, another potential MEKK1 activator (see sect. IVc2) (14). On the basis of these results, it is conceivable that activation of MEKK1 (and, perhaps by extension other MAP3Ks) by Tyr kinases may involve the coordinated recruitment of MEKK1 and its activators to the membrane, followed by their binding to MEKK1 and contribution to its activation (Fig. 8B).
2. Caspases and regulation of MEKK1 during anoikis

Although endogenous, full-length MEKK1 when assayed in vitro displays high basal activity; cleavage of the NH2-terminal regulatory domain results in a modest but significant increase in activity, suggesting that the MEKK1 NH2 terminus exerts an inhibitory effect on activity in the resting state that is relieved by upstream stimuli (344).

Many cells undergo apoptosis upon dissociation from the substratum, a process referred to as anoikis. The apoptosis characteristic of anoikis was recently shown to be regulated in part by MEKK1. There is evidence that during anoikis MEKK1 is regulated by controlled proteolysis of the MEKK1 polypeptide at Asp-871 and Asp-874 (30). This cleavage is catalyzed by caspases. Caspases are a family of at least 11 related cysteine proteases that are vital to the regulation of apoptosis and inflammation. Caspases cleave at Asp residues within the consensus sequence Asp-X-X-Asp (358). Caspase-3 and -7 are activated preferentially in response to apoptotic stimuli, rather than inflammatory episodes, and can be inhibited with the fluoromethyl ketone-derivatized synthetic peptide DEVD-fmk.

Caspase-7 is also inhibited by the cowpox viral protein CrmA (358). A role for caspase-7 and, possibly, caspase-3 in MEKK1 cleavage during anoikis was inferred from studies showing inhibition of MEKK1 proapoptotic activity by DEVD and CrmA. Moreover, caspase-3 and -7 can cleave MEKK1 at Asp-871/Asp-874 in vitro. Caspase cleavage irreversibly disinhibits MEKK1 and apparently unmasks a latent apoptogenic activity. Thus mutant MEKK1 polypeptides wherein the consensus caspase cleavage sites were mutated (Asp-871/Asp-874 → Glu-871/Glu-874) were resistant to activation during anoikis and conferred resistance to apoptosis (30).

Although caspase cleavage renders activation of MEKK1 irreversible, actual activation of MEKK1 likely does not require prior MEKK1 cleavage. Moreover, MEKK1 induction of apoptosis is SAPK independent. Thus expression of caspase-resistant MEKK1 still results in robust SAPK activation. Moreover, activation of the MAP3K activity of MEKK1 occurs before caspase cleavage (338). In addition, kinase-dead SEK1 does not reverse apoptosis incurred upon expression of the free MEKK1 catalytic domain (142). Caspase cleavage of MEKK1 results in a MEKK1 fragment that is free to migrate within the cell. Apparently, this diffusability, and not SAPK activation, is key to MEKK1′s proapoptotic activity (338).

The proapoptotic function of MEKK1 may be unique to anoikis. Thus, although MEKK1 is cleaved by caspases during anoikis, and when ectopically overexpressed, recombinant MEKK1 corresponding to those generated in vivo as a consequence of caspase activity can induce apoptosis, recent genetic studies suggest that the proapoptotic function of MEKK1 is not widespread. mekk1-/- ES cells have been generated. Surprisingly, these cells are more sensitive to proapoptotic environmental stresses (360). Moreover, cardiomyocytes from embryoid bodies derived from MEKK1 knockout ES cells are more prone to undergo apoptosis in response to oxidant stress (209). These findings suggest a protective rather than proapoptotic role for MEKK1.

3. Regulation of MEKK1 by GCKs

Several group I GCKs (GCK itself, HPK1 and NIK) can interact with MEKK1 in vivo and in vitro, and there is evidence that MEKK1 is an effector for these GCKs (131, 294, 359). In all cases, the binding of MEKK1 requires the CTD of the GCK family kinase polypeptide. Although the binding of MEKK1 to the HPK1 CTD has not been mapped in detail, the binding of MEKK1 to GCK and NIK has been well characterized.

NIK binds only full-length MEKK1. Deletion of the first 719 amino acids of MEKK1 abolishes NIK binding, and a truncation construct of MEKK1 consisting solely of amino acids 1–719 will interact in vivo with amino acids 948–1233 of MEKK1. MEKK1 binding has been mapped to the COOH-terminal GCK homology domain (amino acids 948–1233) of NIK, and accordingly, deletion of this domain prevents the binding of MEKK1 (294).

The GCK-MEKK1 interaction is somewhat more complex. GCK can interact in vivo and in vitro with both endogenous or recombinant, full-length MEKK1. One GCK binding site on MEKK1 has been mapped to residues 817–1221 of the MEKK1 polypeptide. Deletion of the kinase domain of GCK or the region of GCK that includes the NH2-terminal PEST domain has no effect on the binding of a MEKK1 construct consisting of amino acids 817–1493. However, deletion of the CT motif of GCK's GCK homology domain abolishes MEKK1[817–1221] binding while subsequent deletion of the Leu-rich region of the GCK homology domain restores binding. Deletion of the COOH-terminal PEST motif of GCK (amino acids 404–447) again abolishes binding to MEKK1[817–1221] but has no effect on the binding of GCK to full-length MEKK1 (D. Chadee and J. M. Kyriakis, unpublished data). These results suggest that amino acids 1–817 of MEKK1 can interact with GCK within a region that includes the middle PEST domain (Fig. 8A) while amino acids 817–1221 require at least the domain spanning the COOH-terminal PEST domain for binding (Fig. 8A). Both full-length and truncated MEKK1 binding to GCK also requires the CT extension of GCK's GCK homology domain which, as described below, may relieve an inhibition to MEKK1 binding conferred by the Leu-rich region (359).

The GCK homology domains of group I GCKs are strikingly conserved. Why, then, does MEKK1 binding to NIK and GCK appear to be so different? First, it is conceivable that the binding of MEKK1 to either GCK or NIK involves multiple contact points both within and outside...
the Leu-rich segments. Indeed, the complex binding of full-length MEKK1 versus MEKK1[817–1221] supports this idea. Second, NIK and the related TNIK are the most divergent of the group I GCKs (163). The Leu-rich region of the NIK and TNIK GCK homology domains are considerably shorter than those of GCK, GCKR, GLK, and HPK1 due to the presence of an additional, highly conserved stretch of ~60 amino acids at the NH₂-terminal end of the GCK, GCKR, GLK, and HPK1 Leu-rich motifs that is absent in NIK and TNIK (73, 96, 131, 145, 151, 159, 163, 277, 294, 319, 354). This extension may perform an inhibitory role that is relieved upon the binding of GCK upstream activators to the CT region. Accordingly, deletion of the CT region, by abolishing the possibility of disinhibition of GCK, would prevent MEKK1 binding.

In this regard, it is noteworthy that full-length, wild-type GCK binds MEKK1 less stably than does the free GCK CTD (359), and kinase-inactive GCK binds MEKK1 even more poorly than does wild-type GCK. Conversely, wild-type GCK is the best in vivo activator of the SAPK pathway (359). Thus activation of GCK could both enable MEKK1 binding and prompt more efficient MEKK1 activation and turnover (163, 359).

It is possible that MEKK1 is an effector for GCK, NIK, and HPK1. In support of this, dominant negative constructs of MEKK1 can block GCK, NIK, and HPK1 activation of SAPK, of note, the GCK-binding fragment of MEKK1 (amino acids 817–1221) (359) but not the SEK1-binding fragment of MEKK1 (amino acids 1221–1493) (342) will inhibit GCK signaling without affecting the kinase activity of GCK (359), suggesting that interfering with the binding of endogenous MEKK1 to GCK inhibits GCK signaling. Exactly how GCK family kinases might activate MEKK1 is still unclear. GCK can phosphorylate MEKK1 in vitro; moreover, the binding of MEKK1 to GCK may allow for the regulated targeting of MEKK1 to site(s) of activation. Alternatively, GCK’s interaction with MEKK1 may foster activation of MEKK1 by an oligomerization-dependent mechanism (see sect. ivC).

4. Binding and activation of MEKK1 by TRAFs

MEKK1 can also interact in vivo with TRAF-2 and -6, in the absence of coexpressed GCKs (although the possibility that endogenous GCKs participate in stabilizing the MEKK1-TRAF2 complex cannot be ruled out, see sect. ivC), and the interaction with TRAF2 is stimulated by TNF and requires the TRAF2 RING effector domain (see sect. iiiE) (13).

Yeast two-hybrid screening and communoprecipitation suggested initially that TRAF proteins could homooligomerize (299). Crystallographic studies indicate that the TRAF1 domains of TRAF2 exist as a trimer when complexed with the trimerized intracellular TRAF binding region of TNFR2 or CD40 (204, 231). This result suggests that TRAFs oligomerize, when complexed with their upstream activators, and it has been suggested that TRAFs are themselves activated to bind their effectors upon oligomerization.

Oligomerization of TRAFs is sufficient to trigger MEKK1 binding. FK506 is a macrolide immunosuppressant that binds to a receptor protein FK506 binding protein-12 (FKBP12). This complex, in turn, can bind to and inhibit the calcium-sensitive phosphatase calcineurin. By this process, activation of NF-ATs during T-cell activation is inhibited, thus accounting for the immunosuppressive activity of FK506 (270). Fusion proteins were prepared consisting of the RING and Zn finger loops of TRAF-2 and -6 linked to the FK506 binding domain of FKBP12. These constructs, when expressed in transfected cells, were forced to oligomerize upon addition of FK1012, a nonimmunosuppressive dimeric analog of FK506 (13). In a manner analogous to the activation of ERK by forced dimerization of FKBP12-Raf-1 (190), these FKBP-TRAF constructs, when expressed in transfected cells, could stimulate SAPK activity and AP-1-driven gene expression upon incubation with FK1012 (13).

MEKK1 associates selectively with oligomerized TRAF2, in vivo in a manner that requires the TRAF2 RING domain and MEKK1 amino acids 1–817. Thus addition of FK1012 to cell expressing FKBP12-TRAF2 results in the formation of insoluble FKBP-TRAF2 aggregates that can be recovered by centrifugation. When MEKK1 is coexpressed with FKBP12-TRAF2, the MEKK1 coprecipitates with the FKBP-TRAF2 in a FK1012-dependent manner. Expression of a truncated FKBP12-TRAF2 construct wherein the NH₂-terminal RING domain is deleted abolishes this coprecipitation. Transiently expressed full-length MEKK1 generally undergoes progressive proteolysis (see sect. ivB2), and it was observed that only full-length MEKK1, and neither recombinant MEKK1[817–1493] nor MEKK1 fragments generated spontaneously in vivo from expressed full-length MEKK1 coprecipitated with FKBP-TRAF2 in an FK1012-dependent manner (13). Given the observation that TRAF2 oligomerizes upon interacting with their upstream activators, it is plausible to suggest that, in vivo ligand-driven TRAF oligomerization may prompt MEKK1 binding. The FK1012-dependent FKBP12-TRAF2-MEKK1 interaction is consistent with this idea.

Further support for the idea that MEKK1 is a TRAF2 effector comes from the observation that transiently expressed MEKK1 and TRAF2 can associate in vivo in a TNF-dependent manner. Again, this association requires the TRAF2 RING domain. Moreover, coexpression of TRAF2 and MEKK1 also results in significant MEKK1 activation (13). These results imply a comparatively simple, linear mechanism whereby TRAF2, through its RING effector domain, directly binds and activates MEKK1. However, as is discussed in the next section, the situation is likely to be more complex.
C. GCKs May Collaborate With TRAFs to Activate MAP3Ks Including MEKK1

The group I GCKs, GCK itself, GCKR, GLK and NIK are activated by TNF in vivo (73, 239, 277, 354), and there is evidence that GCK, GCKR and, possibly, NIK and TNIK (see sect. ivC1) are TRAF effectors (96, 183, 278, 295, 359). In particular, GCKR is activated by TNF and upon coexpression with TRAF2, binds TRAF2 in vivo (277, 278). Furthermore, despite the observation that TRAF2 and MEKK1 can associate apparently independently of coexpressed GCK family kinases (13), there is evidence that GCKs, TRAFs, and, perhaps additional components TNFR family complexes, collaboratively activate MAP3Ks upstream of the SAPKs and p38s. Thus 1) antisense GCKR constructs will block TNF activation of SAPK in 293 cells, and some GCKs associate in vivo with TRAFs (96, 183, 278, 359); 2) dominant inhibitory MEKK1 can block GCK, NIK, HPK1, and GCKR activation of SAPK (131, 277, 295, 359); and 3) genetic studies of Drosophila implicate GCK family kinases as TRAF effectors (see sect. ivC1) (183).

As with MEKK1, GCK, GCKR, and TNIK can bind TRAF2 in vivo. The binding of GCK, GCKR, or TNIK to TRAF2 requires the TRAF domains of TRAF2. The GCK homology domain CT motifs of GCK and GCKR are necessary for TRAF binding (96, 163, 278, 359). In addition, deletion of the NH2-terminal PEST motif of GCK (Fig. 8A) hampers TRAF2 binding (359). Neither GCK nor GCKR possesses a strong consensus TRAF2 TRAF domain binding motif (151, 277, 356) in the regions mapped as necessary for TRAF binding, suggesting that, either the interactions with TRAF2 are indirect or, as with TRADD (356), are direct but involve other motifs.

Coexpression with TRAF2 activates GCKR, and deletion of the TRAF2 RING domain cripples TRAF2's ability to activate coexpressed GCKR (277). From these results, it is plausible to speculate that the TRAF2 TRAF domains bind GCK and GCKR while the RING domains mediate GCK/GCKR activation. Thus a stable interaction with the TRAF2 RING domain is not an absolute requirement for all TRAF2 effectors, although the RING domain is essential for regulation of TRAF effectors.

In contrast, the association of TNK and TRAF2 requires an intermediate segment (amino acids 306–1017) that lies between the kinase domain and the GCK homology domain, while the GCK homology domain appears to be dispensable (96). This result is similar to the binding of the Drosophila group I GCK Missapen (which is closer in sequence to TNIK and NIK than are even the other group I GCKs) to Drosophila TRAF in the dorsal closure SAPK pathway (see sect. ivC1) (295). TNIK possesses four consensus TRAF2 TRAF domain binding sites (TNIK amino acids 326–329, 832–835, 838–841, and 839–842), which are conserved in NIK (96, 295, 356).

A fourth molecular component may also regulate signaling from TRAF2 to GCKs and MEKK1 and may enable the indirect association of TRAF2 and GCK/GCKR. TRAF-associated NF-κB activator (TANK) was isolated in a screen for TRAF3 interactors and was shown to interact with all TRAF isoforms in vivo. A 21-amino acid fragment in the middle of the TANK polypeptide (amino acids 169–190) is required for TRAF binding. At low levels of expression, recombinant TANK synergistically enhances TRAF2 activation of NF-κB, while at higher levels of expression, TANK inhibits NF-κB activation (43), a phenomenon suggestive of a prozone effect (see sect. ivC1), suggesting that TANK might perform a scaffold function. TANK also synergistically increases TRAF2 activation of SAPK, and expression of amino acids 190–413 of TANK, which lie downstream of the TRAF binding domain, can potently block both NF-κB and SAPK activation incurred by TRAF2 (45). Although TANK itself does not interact with GCKR, the interaction between GCKR and TRAF2 is strongly enhanced upon coexpression of TANK, and dominant inhibitory GCKR blocks SAPK activation by coexpressed TRAF2 or TRAF2 plus TANK, suggesting that TANK's stimulation of TRAF activation of SAPK involves stabilization of the TRAF2-GCKR complex (45).

Although biochemical and genetic data (see sect. ivC1) suggest that GCKs either relay signals from TRAFs to MAP3Ks or collaborate with TRAFs to activate MAP3Ks (183, 278, 359), biochemical evidence also indicates that TRAF2 and MEKK1 interact apparently independently of coexpressed GCKs (13). How might these conflicting results be reconciled? It is possible, of course, that both mechanisms operate separately and in parallel. Alternatively, the TRAF2-MEKK1 interaction may be comparatively weak and might be stabilized or potentiated by GCKs once the GCKs themselves are activated by TRAFs. Both the TRAF-MEKK and GCK-MEKK binding results are consistent with the notion that a complex consisting of a group I GCK and MEKK1 could interact in such a way as to permit a regulatory binding of MEKK1 to the TRAF2 RING domain. Another possibility is that both TRAFs and GCKs might contribute contemporaneous, yet independent inputs to MEKK1 activation, requiring the obligatory formation of a TRAF-group I GCK family-MEKK1 complex.

Just what constitutes group I GCK and MEKK1 activation is still nebulous. The results with FKBP-MEKK1 (13) implicate oligomerization in MEKK1 activation, but this has yet to be demonstrated. The fact that GCK's kinase activity enables maximal recruitment of the SAPKs (239, 359) suggests that phosphorylation may also contribute to MEKK1 activation; however, whether or not this might be MEKK1 activating autophosphorylation or phosphorylation catalyzed by GCKs (or, indeed if phosphorylation is at all important to MEKK1 regulation) is somewhat enigmatic. Support for a role for autophosphorylation in the regulation of MEKK1 comes from the finding that the activity of a truncated MEKK1 construct (either amino acids 817–1493 or
1221–1493) requires phosphorylation at Thr-1381 and Thr-1393 for activity. As with many kinase regulatory phosphoacceptor sites, both of these residues lie within the activation loop just upstream of kinase subdomain VIII. Δ1–1221 MEKK1 autophosphorylates at Thr-1381, in vitro, and mutagenesis of either Thr-1381 or Thr-1393 to Ala abolishes activity (65, 282). Moreover, 14–3-3 proteins interact with the NH₂-terminal noncatalytic region of MEKK1 (see sect. nG2). Although the significance of this interaction is unclear, binding of 14–3-3 proteins to their targets requires prior phosphorylation of the target proteins, suggesting that extracatalytic phosphorylation sites on MEKK1 exist (90). What is still unclear is the mechanism by which this MEKK1 phosphorylation is triggered, specifically, is the autophosphorylation cotranslational, with additional steps initiating activation of the full-length protein, or is autophosphorylation triggered by aggregation alone, or are additional phosphorylations of the full-length kinase necessary to disinhibit the autoprophosphorylation mechanism?

As for GCKs, all group I GCKs, as well as JIK contain a conserved Thr residue (Thr-173 for GCK, Thr-178 for GCKR, Thr-175 for HPK1, Thr-164 for GLK, Thr-190 for NIK, Thr-191 for TNIK, and Thr-181 for JIK) which lies just upstream of the Ala-Pro-Glu conserved residues in the subdomain VIII activation loop (73, 96, 151, 159, 277, 294, 303, 319, 354). Mutagenesis of Thr-178 of GCKR to Ala renders GCKR completely inactive, and this construct can dominantly inhibit TNF- and TRAF2 activation of coexpressed SAPK (277). This finding suggests that Thr-178 of GCKR (and, by extension, the analogous residues in the remainder of the GCK family) might be sites that must be phosphorylated for activation. Inasmuch as TRAFs are oligomerized upon interaction with receptors (204, 231), the clustering of GCKs at oligomerized TRAFs may elicit GCK phosphorylation (autophosphorylation?) and activation, triggering MEKK1 binding, and oligomerization-dependent (13) activation (Fig. 10A).

1. *Drosophila* SAPK and the regulation of dorsal closure: parallels with mammalian signaling from TRAFs to GCKs

Analysis of the genetic basis of *Drosophila* morphogenesis has identified a signaling pathway, the dorsal closure pathway, that includes components homologous to the TRAFs and GCKs which recruit a SAPK ortholog (135, 183, 295). These findings support the contention that TRAFs recruit GCKs to signal to the SAPKs. During *Drosophila* embryogenesis, organogenesis and germ band retraction are followed by dorsal closure, the establishment of a contiguous dorsal epidermis. The epidermal primordia of early *Drosophila* embryos is ventral, and the dorsal surface is covered by a primitive tissue, the aminoserosa. Dorsal closure involves a concerted ventrodorsal cell sheet movement that positions and eventually fuses the lateral epidermal primordia over the aminoserosa. This is followed by degeneration of the aminoserosa. Several key components of a *Drosophila* SAPK pathway are necessary for dorsal closure including the products of *basket* (bsk, also called DJNK, a *Drosophila* SAPK), *hemipterous* (hep, a *Drosophila* ortholog of MKK7), *Djun* (*Drosophila* c-Jun), and *Dfos* (*Drosophila* c-Fos). Biochemical and genetic evidence indicate that Hep, Bsk, and DJun are directly linked in a signaling pathway. Additional components include Drac and DCdc42, which, as in mammals, regulate cytoskeletal function and may in part regulate Bsk. The ultimate consequence of DJun activation is expression of *decapentaplegic* (dpp), a member of the TGF-β family that is similar to BMP4. *dpp* is expressed in leading-edge epidermal cells during dorsal closure (135).

*Misshapen* (msn) is a *Drosophila* ortholog of NIK and TNIK (96, 294, 295). Recent studies from Skolnik and colleagues (295) implicate msn in dorsal closure signaling and suggest that Msn acts in parallel with dRac to recruit Bsk. Deletion or mutational inactivation of msn results in dorsal closure defects that are strikingly similar to the *bsk* phenotype (135, 295), and a significant percentage of either doubly heterozygous *msn* −/+, *bskt* −/+ or *msn* −/−, and *hep* −/− flies exhibit a dorsal-open phenotype, with the severity of the phenotype correlating well with the strength of the *bsk* or *hep* allele, findings consistent with the idea that Msn signals to Bsk,. Moreover, constitutively active DJun rescues not only the *bsk* phenotype, but the *msn* phenotype as well. Expression of *msn* in mammalian cells activates coexpressed SAPK (295).

Of particular interest, a *Drosophila* TRAF (*Dtraf*) was recently identified in a yeast two-hybrid screen for Msn interactors. DTRAF also activates coexpressed SAPK in mammalian cells, and kinase-dead Msn can block DTRAF activation of SAPK in mammalian cells (183). DTRAF binds to the Msn CTD at a region of the Msn polypeptide (amino acids 293–587) that corresponds to the region of TNIK that binds TRAF2, and to the domain that includes the PEST1 sequence of GCK (amino acids 293–313), a region which, in addition to the GCK-CT extension, is required for TRAF2 binding (163, 183, 359) (see sect. nV). Although inactivating *dtraf* mutants exhibit a dorsal-open phenotype, genetic evidence placing DTRAF upstream of Msn is not yet available (183). However, these results suggest that a signaling pathway strikingly similar to the TRAF2 →GCK/GCKR →(MEKK1) →SAPK pathway (see sects. nV3 and nV) exists in *Drosophila* to regulate in part dorsal closure (96, 183, 277, 295, 359). Moreover, given the close homology between NIK, TNIK, and Msn, plus the observation that NIK and TNIK are effectors for mammalian TRAFs.
D. ASK1 Is an Effector for TRAFs, Specifically, a TNF Effector Recruited by TRAF2: Role of Cellular Redox in ASK1 Regulation

The coupling of group I GCKs to MAP3Ks and TRAFs provides an attractive model for the regulation of the SAPKs by TNFR family receptors. However, the GCKs, as well as MEKK1 and MLK3 (see sects. II G and III D), are highly selective for the SAPKs, and, therefore, these models do not account for TNFR family regulation of the p38 pathway. Recruitment of ASK1 provides a mechanism for TRAF recruitment of p38.

ASK1 is a MAP3K that can activate both the SAPKs (through activation of SEK1) and the p38s (through M KK3 and M KK6). Moreover, the MAP3K activity of ASK1 itself is activated by TNF (134) (see sect. II G2). ASK1 is also activated upon Fas engagement and may be an effector for Daxx (36).

1. Recruitment of ASK1 by TRAF2 is redox dependent and blocked by reduced thioredoxin

Expression of TRAF-2, -5, or -6 results in vigorous SAPK and p38 activation, and TRAF2 is required for SAPK activation by TNF, while TRAF6 is required for IL-1 activation of SAPK (7, 176, 185, 186, 223, 253, 286, 323, 357, 357),
Recombinant ASK1 can interact in vivo with recombinant TRAF-2, -5, and -6. Moreover, endogenous TRAF2 and ASK1 interact in vivo in a TNF-dependent manner (123, 182, 228). There is some disagreement as to the domains of ASK1 and TRAF2 involved in the ASK1-TRAF2 interaction. Nishitoh et al. (228), Hoeflich et al. (123), and Liu et al. (182) observed that deletion of the TRAF2 TRAF domains (especially TRAF-N) seriously compromises ASK1 binding. However, whereas Nishitoh et al. (228) and Liu et al. (182) observed that the free TRAF domains of TRAF2 can bind ASK1, Hoeflich et al. (123) observed no interaction between ASK1 and the free TRAF2 TRAF domains. Moreover, Hoeflich et al. (123) observed that the TRAF2 RING effector domain may be important to ASK1 binding and found that deletion of this domain also substantially reduces ASK1 binding, a phenomenon not observed by Nishitoh et al. (228) or Liu et al. (182). The basis for these differences is unclear and cannot be attributed to cell type, transfection conditions, or choice of plasmid.

Both Nishitoh et al. (228) and Liu et al. (182) observed that the COOH-terminal noncatalytic domain of ASK1 (amino acids 937–1375) interacts with TRAF2 (through the TRAF domains). However, Liu et al. (182) also observed that the NH2-terminal 460 amino acids of ASK1 can interact with the TRAF domains of TRAF2. Moreover, coexpression of this fragment of ASK1 blocks TRAF2 and TNF activation of SAPK, suggesting that this domain is relevant to ASK1 activation by TRAF2.

Coexpression of ASK1 and TRAF2 also results in activation of ASK1 (182, 228). Given that ASK1 is a potent activator of p38 (134), the TRAF2-ASK1 interaction might also mediate the activation of p38 by TNF.

The regulation of ASK1 by TRAF2 is redox dependent (Fig. 10B). Thus addition of free radical scavengers prevents the TNF-dependent association of endogenous ASK1 and TRAF2 (182). Yeast two-hybrid screening has revealed that the redox sensing oxidoreductase thioredoxin (Trx) is an endogenous inhibitor of ASK1. This inhibition requires that Trx be in a reduced state. Thus treatment of cells with oxidant stress (H2O2) triggers the dissociation of Trx from ASK1 and the activation of ASK1 in vivo (263). TNF can trigger the release of reactive oxygen intermediates (ROIs); however, the biochemical mechanism by which this process is regulated is still nebulous, especially in nonphagocytic cells. TNF treatment is known to generate a mitochondrial pulse of reactive oxygen intermediates (ROIs) with kinetics (maximal 1 h) even slower than the comparatively slow activation of ASK1 by TNF (detectable within 10 min and maximal at 20 min) (107, 108). An additional more rapid onset ROI pulse of unknown origin may be generated upon recruitment of TRAF2 inasmuch as activation of the SAPKs by coexpressed TRAF2 is partially reversed with free radical scavengers (223), and expression of TRAF2, but not a mutant TRAF2 construct missing the RING effector domain, triggers the production of ROS (182). TNF fosters the dissociation of ASK1 from Trx by a process that can be blocked with free radical scavengers (263).

Recent results suggest that Trx sequesters ASK1 from TRAF2 until TRAF2-dependent ROS production stimulates the dissociation of the ASK1-Trx complex (182) (Fig. 10B). Trx is abundant in cells and serves as a catalytic antioxidant (125, 126). In contrast, TRAF2 and ASK1 are low-abundance polypeptides (134, 257). Expression of Trx at levels in excess of coexpressed TRAF2 and ASK1 completely reverses the ASK1-TRAF2 interaction. The interaction between ASK1 and TRAF6 is also reversed upon coexpression of excess Trx. The inhibition of the ASK1-TRAF2 interaction can be reversed upon the subsequent administration of oxidant (H2O2) (182). Taken together, these results suggest that TRAF2 engagement by the TNFR complex triggers the production of ROS, which oxidize Trx causing its dissociation from ASK1, thereby enabling the binding of TRAF2 to the free ASK1 polypeptide. How is it possible, then, to observe an interaction between overexpressed ASK1 and TRAF2, in the absence of excess Trx, without addition of oxidant? It is likely that overexpression of ASK1 and TRAF2 enables the ASK1-TRAF2 interaction against a background of endogenous Trx 1) because the overexpressed ASK1 titers out endogenous Trx, creating a pool of free ASK1 to which TRAF2 can bind, and/or 2) overexpression of TRAF2 generates a ROS pulse in vivo resulting in oxidation of endogenous Trx, thereby enabling the ASK1-TRAF2 interaction (182).

A likely consequence of Trx dissociation from ASK1 is dimerization-dependent ASK1 activation. Upon overexpression, ASK1 spontaneously dimerizes in vivo, and TNF promotes the dimerization of endogenous ASK1 by a mechanism that requires ROS and can be reversed with free radical scavengers (108). Moreover, expressed fusion proteins of ASK1 and DNA gyrase can be forced to dimerize in vivo upon administration of the binary DNA gyrase-binding drug coumermycin. This coumermycin-induced dimerization results in substantial activation of coexpressed SAPK (108). TRAF2 is known to homodimerize in vivo (204, 231, 299), and coexpression of ASK1 and TRAF2 enhances the level of recoverable ASK1 homomers in a reaction that requires the TRAF2 RING domain (i.e., is not observed upon expression of TRAF2ΔRING) and is blocked by free radical scavengers (182) (Fig. 10B).

2. ASK1 recruitment by Daxx conflicting results

As was noted in section 11F5, Daxx is a novel adapter protein that couples Fas to SAPK activation. In certain cell types, Daxx-induced apoptosis requires activation of the SAPKs (352). Chang et al. (36) observed that Fas engagement could activate endogenous ASK1. These investigators also observed that coexpression of Daxx and
ASK1 results in substantial activation of ASK1. Moreover, both endogenous and recombinant Daxx can associate directly in vivo and coimmunoprecipitate with ASK1 in a reaction that is Fas dependent and requires the NH₂-terminal 648 amino acids of ASK1. Kinase-inactive ASK1 effectively blocks both Daxxx-induced apoptosis and SAPK activation (36). Thus ASK1 is a putative downstream target of Daxx that couples Fas to the SAPKs.

A critical caveat of these findings comes from recent examinations of the subcellular localization of Daxx. As was mentioned in section mF5, Daxx has been isolated in yeast two-hybrid screens as an interactor with nuclear proteins such as DNA methyltransferase (208). Moreover, in apparent contrast to the ASK1 coimmunoprecipitation results described above (36), anti-Daxx antisera were used in cell fractionation studies to demonstrate a predominantly nuclear localization for Daxx (208, 313).

E. TAK1 Is a Target for TGF-β and IL-1 Through Its Association With TAB1

TAK1 was cloned as part of a screen to isolate novel mammalian MAP3Ks that could rescue S. cerevisiae deficient in STE11, a MAP3K of the mating factor pathway (346) (see sect. iiG2). TAK1 can recruit both the SAPKs (via activation of SEK1) and the p38s (via activation of MKK3 and MKK6). Substantial evidence suggests that TAK1 couples the SAPKs to the TGF-β superfamily receptors (217, 346). The TGF-β superfamily [TGF-β, the activins, bone morphogenic proteins (BMPs) and others] regulates numerous morphogenic and developmental processes. Signaling by TGF-β superfamily receptors has been reviewed elsewhere (9, 199).

TGF-β and BMP-2 and -4 can activate the SAPKs in some cell lines; the MAP3K activity of TAK1 itself is activated by TGF-β and BMP-4 in murine osteoblastic cells (MC3T3-E1). Moreover, transient ectopic expression of TAK1 can stimulate trans-activation of reporter genes driven by the TGF-β-sensitive plasminogen activator inhibitor-1 (PAI1) promoter (217, 346, 345). The small NH₂-terminal extension of TAK1 plays an inhibitory role. Thus, in resting cells, recombinant, ectopically expressed TAK1 is inactive; however, deletion of the NH₂-terminal 22 amino acids from the TAK1 polypeptide results in constitutive activation (346).

TAK1 binding protein-1 (TAB1) is an essential cofactor required for activation of TAK1 by upstream stimuli. A 55-kDa polypeptide with no obvious structural features indicative of biochemical properties, TAB1 was cloned in a two-hybrid screen for interactors with the NH₂-terminal 22-amino acid regulatory domain of TAK1 (279). Coexpression of full-length TAB1 with TAK1 results in TAK1 activation, assayed using the S. cerevisiae STE11 rescue assay (see sect. iiG2), or in transfected cells using either the PAI1 reporter system or p38 activation as a readout (279).

Recently, a yeast two-hybrid screen to identify novel TAB1 interactors identified the human X chromosome-linked inhibitor of apoptosis (XIAP) as a TAB1 binding protein (345). XIAP is a member of an emerging family of polypeptides, the inhibitors of apoptosis (IAPs). IAPs were originally identified as baculoviral encoded proteins and were subsequently shown to exist in mammalian cells where they function as inhibitors of proapoptotic signaling from TNFR family receptors. All IAPs contain three baculoviral IAP repeat (BIR) motifs followed by a COOH-terminal RING domain (71). The NH₂-terminal-most of the three XIAP BIR motifs is required for binding TAB1, while the RING domain is required for interacting with type I BMP receptors (345). Given that different domains of XIAP interact with TAB1 and BMPR type I, it is not surprising that trimeric complexes of BMPR-I, XIAP, and TAB1 can be isolated (345). XIAP also interacts directly with the type I receptors for BMP-2 and -4. The physiological significance of the XIAP-TAB1-TAK1 complex was demonstrated when it was observed that XIAP expression in Xenopus embryos could mimic the ventralizing effects of BMP, while expression of the TAB1 binding region of XIAP (see below) could block the expression of ventral mesoderm markers elicited by a constitutively active mutant type I BMP receptor (345).

Other members of the IAP family can be recruited to TNFR complexes. Thus cIAP1 and cIAP2 interact with TRADD and TNFR1 (71). The interaction between XIAP and TAB1 raises the possibility that IAPs may serve as adapters that recruit MAP3Ks to receptors of the TNFR family. In this regard, a recent study has also implicated TAK1 in IL-1 signaling to the SAPKs. Thus both IL-1 and coexpressed TRAF6 can activate TAK1. In the latter instance, TAB1 is required for activation (Fig. 10C). Moreover, coexpressed TRAF6 and TAK1 can coimmunoprecipitate in a reaction that requires TAB1 (225). The mechanism by which TRAF6 activates TAK1/TAB1 is unknown.

F. MEKK4 Is a Putative Effector for Rho Family GTPases and May Be Activated by DNA Damage Through a Direct Interaction With GADD45 Homologs

1. MEKK4 is a possible effector for Rho GTPases

MEKK4, a MAP3K that can activate both the SAPK pathway (through activation of SEK1) and the p38 pathway (through MKK3 and MKK6) (103, 297) (see sect. iiG2), possesses a putative CRIB motif in its NH₂-terminal regulatory domain (amino acids 1311–1324). Consistent with this, there is some evidence that MEKK4 may be a target for Rho family GTPases (89, 103). MEKK4 can bind Rac1 and Cdc42Hs in vitro and in vivo; however, these interactions appear to be GTP independent (89, 103). Moreover, while kinase-inactive MEKK4 can inhibit activation of SAPK by constitutively
active Rac1 and Cdc42Hs, as with MEKK1 (89), there are no data indicating whether or not the activity of MEKK4 is actually modified as a consequence of binding Rac1 or Cdc42Hs. In the absence of these data, results using kinase-inactive MEKK4 need to be interpreted cautiously as these dominant inhibitors may merely sequester downstream effectors shared by MEKK4 and other MAP3Ks.

2. Recruitment of MEKK4 by ionizing radiation and other genotoxic stresses

Most chemotherapeutic and radiation treatment protocols employed to combat cancer kill cells by eliciting irreversible DNA damage and consequent apoptosis of tumor cells. An unwanted side effect of these treatments is the collateral killing of normal cells. Accordingly, there is a considerable need to understand how genotoxins trigger cell death so as to enable the improvement of conventional anticancer treatments.

In general, genotoxic stress triggers rapid cell cycle arrest, and, to maintain genomic integrity, either the initiation of DNA and general cellular repair, or, if the damage is too great, apoptosis. Genotoxins such as UV and γ-radiation, methylmethane sulfonate (MMS), cytosine arabinoside (AraC), N-acetoxy-2-acetylaminofluorene, cis-platinum (CDDP), and mitomycin-C have in some instances been shown to activate the SAPKs, p38s, and other stress-activated signaling pathways (41, 70, 157, 184, 230, 273). However, attempts to identify the spectrum of genotoxic stresses capable of activating the SAPKs, and to identify the upstream components that couple these stresses to the SAPKs, have yielded conflicting results.

Activation of the SAPKs by UV radiation (most studies have employed UV-C, a component of the UV spectrum that does not penetrate the Earth’s upper atmosphere) is rapid, and it is generally agreed that UV signaling to protein kinase cascades does not require DNA damage per se. Instead, UV triggers the clustering and activation of cell surface mitogen and cytokine receptors, a process likely driven by the formation of disulfide bridges between adjacent receptor proteins (256).

In contrast, activation of the SAPKs by γ-radiation and most chemical genotoxins proceeds slowly, and most investigators believe that recruitment of the SAPKs by these stimuli requires DNA damage as a triggering event (41, 157, 180, 230). Thus, for example, the ataxia telangiectasia mutated (ATM) gene product, a polypeptide implicated in the cellular response to DNA damage, is necessary for SAPK activation in response to a number of radiant and chemical genotoxins (93, 149, 273).

In addition to ATM, the Tyr kinase protocoprotein c-Abl has been implicated in signaling from DNA damage to the SAPKs and p38s. In this instance, however, there is considerable disagreement among investigators as to the degree of SAPK activation incurred by different genotoxins and the role played by the Abl Tyr kinase in genotoxic signaling. Thus Kharbanda et al. see strong activation of the SAPKs by γ-radiation and chemical genotoxins (CDDP, mitomycin-C) that apparently requires c-Abl and does not occur in abl −/− mouse fibroblasts. These investigators were able to restore genotoxin activation of the SAPKs into abl −/− cells upon reintroduction of c-Abl cDNA (157, 230). In contrast, Liu et al. (184) observed at best modest SAPK activation by γ-radiation and CDDP and substantial activation by mitomycin-C; however, activation of the SAPKs by these stimuli was unaffected in fibroblasts wherein c-abl was disrupted (184). The reason for this discrepancy is unclear, as is the relationship, if any, between ATM and Abl in signaling from DNA damage to the SAPKs. Moreover, these studies do not indicate which MAP3Ks couple the SAPKs to genotoxin signals.

A series of recent studies provides some possible clues as to the identity of the MAP3K → MEK → MAPK core pathways recruited by genotoxins. As with the abl −/− studies, however, there have been conflicting findings. Results from Saito and co-workers (298) indicate that MEKK4 is recruited by genotoxins that transcriptionally induce proteins of the GADD45 family. GADD genes are a set of transcripts that are rapidly induced by DNA damage and are thought to play a role in coordinating the cellular response to genotoxins (93, 149). GADD153/CHOP, described in section II of this paper, is one example. Gadd45 was one of the first GADD genes to be identified, although its function has remained obscure. Expression of GADD45 is the culmination of a signaling pathway that requires prior expression of the tumor suppressor protein p53 which trans-activates the gadd45 (gadd45α) gene. p53 expression is, in turn, driven by the activity of the ATM gene product (149). This is particularly noteworthy given the observation, cited above, that cell lines derived from AT patients with null or inactivating ATM mutations are resistant to SAPK activation by genotoxins but not by TNF (273).

Yeast two-hybrid screening using MEKK4 as bait identified three gadd45 homologs (GADD45α, which is the original GADD45, plus GADD45β and -γ) as direct MEKK4 interactors. These results were confirmed in experiments wherein MEKK4 was overexpressed with recombinant GADD45s, and GADD45γ is the strongest MEKK4 interactor of the three GADD45 isoforms. A broad segment in the middle of the GADD45 polypeptides (amino acids 24–147 of GADD45γ) binds to a small domain (amino acids 147–215) at the NH2-terminus of the MEKK4 polypeptide (298).

Evidence favoring a role for GADD45s in genotoxic regulation of MEKK4 signaling to the SAPKs and p38s comes from the observation that all three gadd45 genes are transcribed in response to the same genotoxic stimuli shown to upregulate gadd45α (93, 298). Moreover, Takekawa and Saito (298) observed that ectopic overexpression of all three GADD45 polypeptides results in substantial activation of both the p38 and SAPK, a reaction that can be reversed upon coexpression with kinase-dead
MEKK4. Kinase-dead MEKK4 can also inhibit activation of p38 by genotoxic stress that transcriptionally induce gadd45 gene expression. In addition, expression of all three GADD45 polypeptides activates MEKK4 in vivo (298). Most importantly, however, addition of purified, recombinant GADD45 proteins to immunoprecipitated MEKK4 activates MEKK4 in vitro, suggesting that the GADD45s are direct MEKK4 activators (298).

Ectopic overexpression of GADD45 proteins is apotelogenic in HeLa cells, a reaction that may require in part GADD45a insofar as either kinase-dead MEKK4 or the GADD45 binding domain of MEKK4 (amino acids 147–250) significantly blocks GADD45-induced apoptosis, while an in-frame deletion of the GADD45 binding domain of MEKK4 renders kinase-dead MEKK4 incapable of blocking apoptosis (298).

Taken together, these results implicate GADD45s as activators of the SAPKs, p38s, and apoptosis via an MEKK4-dependent mechanism. The requirement for GADD45 transcription also explains the slow kinetics of SAPK and p38 activation by many genotoxins (41, 157, 184, 230). The fact that SAPK activation by genotoxins is ablated in ATM null or mutant cells (273) can also be explained by the observation that GADD45 expression (and, hence, subsequent activation of the SAPKs) is also blunted in these cells (149).

However, although expression of GADD45s is up-regulated by stimuli that can activate the SAPKs, missing from the studies implicating MEKK4 in coupling genotoxic stress to the SAPKs are experiments showing that GADD45 proteins are present at times when MEKK4 is activated, and contemporaneously with SAPK activation. In this regard, recent findings apparently conflict with the observation that DNA damage activates SAPK through GADD45α and, perhaps, other GADD45 homologs. Thus studies from Shaulian and Karin (276) as well as Holbrook and co-workers (324) indicate that none of the endogenous GADD45 homologs is present at detectable levels when SAPK is fully activated by DNA damaging chemicals (MMS). Moreover, gadd45α knockout cells show adequate SAPK activation by MMS and other genotoxic stresses, although this experiment does not rule out the possibility that this SAPK activation is mediated by GADD45β or -γ, both of which are also induced by MMS. Still, incubation with cycloheximide (employed at concentrations too low to activate SAPK) to block induction of all GADD45 isoforms also fails to prevent MMS activation of SAPK (276). In contrast to the results of Saito, Holbrook and co-workers (324) do not observe activation of SAPK by coexpressed GADD45a, -b, or -γ. The results of Holbrook and colleagues may reflect the effects of modest GADD45 expression, whereas greater levels of GADD45 may promote SAPK activation (298). However, it is unclear if, under physiological circumstances, such high levels of GADD45 are attained. Even so, in fibroblasts (p53 positive), treated with ionizing radiation sufficient to induce GADD45 expression, there is little or no SAPK activation (276). It must be borne in mind, however, that while a role for GADD45s in the regulation by genotoxins of the SAPKs (via MEKK4 or otherwise) is controversial, there may be physiological circumstances, such as certain developmental stages, during which GADD45s are expressed under conditions wherein they can recruit MEKK4, and through MEKK4, the SAPKs, and p38s.

G. Regulation of MLK3 by Group I GCKs and Cdc42Hs: Role of Dimerization Through the Leu Zippers

1. Regulation of MLK3 by the group I GCK HPK1

There is evidence that the mixed lineage kinase MLK3 (see sect. μG3) is an effector for HPK1 (159) (see sect. μD2). Thus not only can HPK1 associate in vivo with MEKK1, but MLK3 was identified in a yeast two-hybrid screen for HPK1 interactors (159, 309). HPK1 binds MLK3 in an interaction that requires the NH2-terminal SH3 domain of MLK3 (amino acids 1–93) and the COOH-terminal two of the four SH3 binding domains of HPK1 (amino acids 433–442 and 468–474) (159). Expression of kinase-dead MLK3 abrogates activation of SAPK by coexpressed HPK1, suggesting that MLK3 is an HPK1 effector (159). In contrast, the NH2-terminal SH3 binding domains of HPK1 interact with Grb2 and Crk/CrkL. Crk/CrkL activates HPK1 in vivo (181) (see sect. μD2).

2. Regulation of MLK3 by Cdc42Hs

Although the role of Rho family GTPases in the regulation of MEKK1 and MEKK4 is still nebulous (see sect. v, B1 and G1), MLK3 and MLK2, two mixed lineage kinase activators of the SAPKs, contain CRIB motifs and can interact directly with Cdc42Hs and Rac1 in a GTP-dependent manner.

Although MLK-2 and -3 may be Cdc42Hs effectors (below), it is unlikely that they are Rac targets (222, 302). Thus Phe37Ala-Rac1 is an effector loop mutant of Rac1 that retains its ability to recruit the SAPK pathway in vivo (168). However, neither MLK3 nor MLK2 can interact with Phe37Ala-Rac1 in vivo either in COS1 cells or in a yeast two-hybrid assay (168, 302). As discussed in section μA2, Phe37Ala-Rac1 can bind POSH, and POSH is a likely candidate effector coupling Rac1 to the SAPKs (302).

In contrast, there is evidence that MLK3 is a Cdc42Hs effector. As with all MLKs (see sect. μG3), MLK3 possesses a leucine zipper (amino acids 400–487) (101). Leucine zippers frequently mediate protein-protein binding; for example, the bZIP transcription factors in AP-1 dimerize via leucine zipper interactions. MLK3, when overexpressed, will spontaneously dimerize in vivo. The leucine zipper domain of MLK3 is required for this ho-
modimerization, insofar as deletion of this domain abrogates in vivo MLK3 homodimerization (180).

The MLK3 leucine zipper is also critical for signal transduction. Deletion of this domain, even in the presence of a fully competent kinase domain, completely inhibits the ability of MLK3 to recruit the SAPK pathway in vivo and converts the truncated construct into a dominant inhibitor of wild-type MLK3 signaling. Of particular importance, coexpression of MLK3 with Val12-Cdc42Hs substantially enhances the ability of MLK3 to homodimerize (180). From these results, it is plausible to speculate that GTP-Cdc42Hs binds and promotes MLK3 (or MLK2) homodimerization and activation.

Grb2, Crk, and CrkL can mediate the mitogen-stimulated recruitment of HPK1 to autophosphorylated Tyr kinase receptors at the plasma membrane. Different regions of the HPK1 polypeptide mediate the interactions with SH2/SH3 adapters and MLK3 (5, 181), and one function of the recruitment of HPK1 to the membrane might be to position associated MLK3 in the vicinity of GTP-Cdc42, fostering MLK3 dimerization and activation. Alternatively, HPK1 (and GCK) may bind MLK3 and foster its dimerization-dependent activation independently of Cdc42Hs.

V. BIOLOGICAL FUNCTIONS OF THE STRESS-ACTIVATED PROTEIN KINASE AND P38 PATHWAYS IN THE STRESS RESPONSE, CELL CYCLE CONTROL EMBRYOGENESIS, AND IMMUNE SYSTEM MATURATION

A. The SAPKs Can Participate in Regulating Apoptosis in Response to Environmental Stress Through AP-1-Regulated Transcriptional Induction of FasL

Withdrawal of NGF or nutrients from differentiated PC-12 cells or primary cultures of neurons results in apoptosis, and there is some evidence that this apoptotic program requires the SAPKs and p38s (178a, 341). Thus NGF withdrawal-induced apoptosis in PC-12 cells correlates with the activation of the SAPKs and p38s and a decrease in ERK activity. Conversely, overexpression of constitutively active MEKK1 in PC-12 cells both activates the SAPKs and promotes apoptosis even in the presence of NGF (341). Similarly, constitutively active forms of MKK3, when overexpressed with p38, can promote apoptosis in the presence of NGF. Reciprocally, and in apparent contrast to fibroblasts where SEK1-K129R fails to block MEKK1-induced apoptosis (142) (see sect. vB2), nonphosphorylatable, dominant inhibitory mutants of c-Jun can prevent MEKK1-induced PC-12 cell apoptosis, and the expression of dominant interfering mutants of MKK3 can prevent apoptosis incurred by NGF withdrawal (178a, 341). Insofar as a constitutively active, oncogenic form of MEK1 can prevent apoptosis induced by NGF withdrawal, it has been suggested that the decision to initiate apoptosis in PC-12 cells depends at least in part on the balance of SAPK and p38 versus ERK activity (341).

Insight into the mechanism by which the SAPKs might promote apoptosis in response to nutritional/trophic factor withdrawal or genotoxic stress came from the results of Green and colleagues (148). Jurkat cells treated chemotherapeutic genotoxins (etoposide, tenoposide) or UV irradiation rapidly undergo apoptosis coincident with the transcriptional induction and cell surface expression of FasL (148). The binding of newly expressed FasL to existing constitutively expressed Fas triggers an autocrine apoptotic program in response to these genotoxins. The promoter for FasL contains AP-1 and NF-κB cis-acting elements, and inhibition of AP-1 activation (with dominant inhibitory SAPK to block AP-1 activation or with a dominant negative c-Jun construct, TAM67, in which the trans-activating δ-domain is deleted) prevents FasL induction (148). Similarly, overexpression of a non-degradable IκB mutant prevents FasL induction. Taken together, these results indicate that genotoxic stresses promote apoptosis by recruiting the SAPKs and NF-κB activation machinery which, in turn, activate AP-1 and NF-κB-mediated induction of FasL (148). Induction of FasL is not restricted to genotoxic stresses. Nutrient withdrawal in cultured cerebellar granule neurons, or NGF withdrawal PC-12 cells, also results in induction of FasL by a SAPK-dependent mechanism (178a).

Studies of heat shock-dependent apoptosis have revealed both a role for the SAPKs in this process and a novel mode of SAPK regulation. TR-4 cells are a thermo-tolerant subline of the murine fibroblast line RIF-1. Comparative studies of the apoptogenic effects of heat shock and genotoxic (cisplatinum) stress on these cells has implicated the SAPKs in the regulation of stress-induced TR-4 or RIF cell death (362). Whereas TR-4 and RIF-1 cells express identical amounts of SAPK polypeptide, TR-4 cell SAPKs are not activated by heat shock, despite the ability of UV radiation to activate of the SAPKs in both cell lines. Furthermore, whereas heat shock and cisplatinum readily kill RIF-1 cells, these treatments are not apoptogenic in TR-4 cells; RIF-1 and TR-4 cells, however, are equally sensitive to UV-C radiation (362). This finding is consistent with the observation that deletion of sek1 abolishes heat shock activation of SAPK (226) (see sect. nF1). Moreover, if the thermosensitive Rif1- cells are stably transfected with a mutant of Sek1, wherein the phosphoacceptor sites are changes to nonphosphorylatable residues (SEK-Ser257Ala/Thr261Leu, SEK-AL), the activation of SAPK, but not p38, to all stimuli is inhibited. Rif-1 cells expressing SEK-AL acquire resistance to the cytocidal effects of UV-C as well as to heat shock and cisplatinum, indicating that activation of the SAPK pathway is required...
for efficient cell death induced by these stress agonists (362).

Heat shock activation of the SAPKs appears to bypass the “traditional” three-tiered core module. Although SEK1 is required for heat shock activation of SAPK (226) (see sect. aF1), SEK1 is not actually activated by heat shock per se. Instead, heat shock suppresses a Tyr phosphatase that ordinarily inactivates SAPK, thereby allowing basal SEK1 Tyr kinase activity (SEK1 and SAPK activity is never entirely absent, even in the resting state) to slowly activate SAPK (206). A consequence of heat stress is the transcriptional induction of the chaperone protein HSP70. HSP70 expression reactivates this SAPK-targeted Tyr phosphatase resulting in SAPK inactivation (206). In this regard, it is noteworthy that TR-4 cells constitutively express high levels of HSP70, and HSP levels are not transcriptionally regulated by heat stress, accounting perhaps for the inability of heat shock to activate SAPK in these cells (362).

B. The p38s Can Trigger Cell Cycle Arrest at G1/S as Part of a Signaling Pathway in Late G1, Mediated by Cdc42Hs and/or MEKK3

The Rho family GTPases RhoA, Rac1, and Cdc42Hs are required for the reentry of quiescent G0 cells into the G1 stage of the cell cycle transition. In addition, Rho family GTPases are important effectors required for the oncogenicity of transforming alleles of ras. Thus microinjection, in G0, of constitutively active mutants of Rac1 or Cdc42Hs can prompt quiescent Swiss 3T3 fibroblasts to transit from G0 into G1; and continuous, ectopic expression of Cdc42Hs can transform NIH3T3 and Rat-1 fibroblasts. Moreover, inhibition of Cdc42Hs or Rac1 significantly reduces Ras-mediated transformation of Rat-1 fibroblasts (229, 245, 246).

However, in cells already committed to the cell cycle, Cdc42Hs, signaling through p38, in contrast to its role in quiescent cells, may have a role in cell cycle inhibition (213). Thus microinjection of p38a, MKK3, or MKK6, but not SAPK, into NIH3T3 cells synchronized in early G1 (with expression first detectable in mid G1) arrests cells at the G1/S boundary. p38a-induced cell cycle arrest is prevented by kinase-dead MKK3, implicating active p38 in this process (213). Expression of Cdc42Hs, but not Rac1 or RhoA, in early G1 also potently arrests cells at G1/S in several cell lines (NIH3T3 fibroblasts, Mv1-Lu mink lung epithelial cells, and MG63 osteosarcoma cells). The Cdc42Hs-induced growth arrest of G1-committed cells can be effectively blocked with dominant inhibitory, kinase-dead MKK3, again implicating the p38s in Cdc42Hs-mediated cell cycle arrest (213). Thus Cdc42Hs serves as a transforming/growth-promoting protein in quiescent cells, or as a growth suppressor in cells already committed to the cell cycle. Inasmuch as p38 is a common target of Rac1 and Cdc42Hs, different Cdc42Hs targets may collaborate with p38 in G1/S growth arrest versus growth promotion.

The mechanisms by which Cdc42Hs couples to p38 to inhibit the cell cycle are unknown. A noteworthy candidate is MEKK3. Thus Siebenlist and colleagues (82) observed that expression of an estrogen-inducible estrogen receptor MEKK3 fusion construct blocks cell proliferation incurred by activated Ras (82).

C. The SAPKs and Brain Development

Gene disruption studies have revealed specific roles for the different SAPK isoforms in the brain. These studies reveal functions related to stress responses and brain development.

Kainic acid is a glutamate receptor agonist that rapidly induces seizures similar to those of epilepsy. In addition, kainate also causes widespread hippocampal neuronal apoptosis similar to that which occurs in stroke. Whereas SAPKα/JNK2 and SAPKβ/JNK1 are ubiquitously expressed, SAPKβ/JNK3 is selectively expressed in brain, heart, and testis. Flavell and colleagues (348) disrupted the gene for SAPKβ in mice and observed that the knock-out animals were markedly resistant to kainate seizures. More importantly, however, deletion of sapkβ protected these animals from kainate-induced neuronal apoptosis (348). Hippocampal cell death is a major clinical indicator in stroke and Alzheimer’s disease. It will be important to determine if ischemia or Alzheimer’s-induced cell death correlates with SAPKβ activation. It will also be important to determine if hippocampal cell death induced by kainate involves upregulation of FasL (see sect. aV4).

The establishment of a role for SAPKβ in the brain’s response to stress prompted the investigation of the role of the SAPKs in normal brain development and function. Kuan et al. (161) and Sabapathy et al. (262) generated mice with sapkα/JNK2, sapka/sapkβ, and sapky/sapkα double knockouts. Studies of these mice revealed a pivotal role for the combined effects of SAPKα and -γ in regional-specific apoptosis during early brain development (161, 262). In contrast, inasmuch as the sapky/sapkβ and sapka/sapkβ animals were viable, deletion of sapka or sapky alone is insufficient to affect brain development, and SAPKβ likely is not a rate-limiting component in brain development, but, as described above, may serve in the response to stress (348).

The sapky/sapka double knockout was embryonic lethal and resulted in an abnormally prominent hindbrain exencephaly. Normally, the closure of the hindbrain neural tube is an essential step in the process of cephalic neurulation. Cell death is important to hindbrain neural tube closure. However, compared with wild-type or single
knockouts, sapkγ/sapka double knockouts exhibited reduced pyknotic nuclei at the lateral edges of the converging hindbrain, an indication of reduced apoptotic cell degeneration (161, 262). Thus SAPKα and -γ collaborate to foster appropriate developmental hindbrain apoptosis.

In contrast, the sapkγ/sapka double knockout, while decreasing hindbrain developmental apoptosis, increased forebrain apoptosis [assayed as pyknotic nuclei or terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) positive cells, a measure of apoptotic internucleosomal cleavage], suggesting that the SAPKs suppress apoptosis in the forebrain (161, 262). This increased apoptosis was attributed to excess activation of caspase-3, as determined by an observed increase in staining with the CM1 antibody which recognizes the cleaved, 17-kDa active form of caspase-3, but not its inactive 35-kDa precursor (161, 262). Thus SAPKα and -γ together exert opposing effects with regard to fore- and hindbrain developmental apoptosis. The basis for this difference is nebulous. It is conceivable that the cell context, SAPKα and -γ substrates present, or the spectrum of pathways activated in conjunction with SAPKα and -γ during development determines the ultimate effects of SAPKα and -γ on apoptosis.

D. The SAPKs and p38s Are Required for the Progression of Cardiomyocyte Hypertrophy in Response to Pressure Overload and Vasoactive Peptide

Pressure overload cardiac hypertrophy (a consequence of hypertension) and cardiomyocyte apoptosis and necrosis (arising from ischemic injury) are the leading causes of cardiovascular morbidity and mortality in the developed world. At the cellular level, hypertension subjects cardiomyocytes to mechanical shear stress. In addition, hypertension elicits the release of the vasoactive peptides endothelin-1 and ANG II. Hypertensive mechanical strain, acting directly, and through vasoactive peptides, in turn, can elicit cardiomyocyte hypertrophy. Cardiomyocyte hypertrophy is marked by cell enlargement, increased protein synthesis (as a function of DNA synthesis), as well as the formation of rigid cytoskeletal actomyosin fibrillar networks and the expression of embryonic genes such as atrial natriuretic factor (ANF). Cardiomyocyte contractility is compromised as a result of these changes, leading ultimately to heart failure (92). Recent results from Olson and co-workers (212) have implicated the Ca2+/calmodulin-dependent phosphatase calcineurin (CaN/PP2B), signaling through NF-AT3, in cardiac hypertrophy. However, the situation is likely to be much more complicated.

Chien and colleagues (329, 330) have observed that markers of cardiomyocyte hypertrophy (cell enlargement, actomyosin fibrils, and ANF expression) could be induced in cultured neonatal rat cardiomyocytes upon ectopic expression from recombinant adenoviruses of constitutively active forms of MKK6 and MKK7. Force and colleagues (46) extended these results to known physiological activators of cardiomyocyte hypertrophy. Thus expression of kinase-dead Lys129Arg-SEK1 from recombinant adenoviruses can block completely cardiomyocyte hypertrophy (cell enlargement, actomyosin fibrils, and ANF expression) incurred in neonatal rat cardiomyocytes by endothelin-1 (46).

E. The SAPKs, p38s, and Ischemic/Ischemic-Reperfusion Injury

A potential role for the SAPKs and p38s in the pathogenesis of ischemic injury came with the initial observation that the SAPKs were activated selectively during the reperfusion of ischemic kidney (237). Subsequently, it was demonstrated that the same held true for reperfused heart (22). In contrast, the p38s are activated during ischemic injury and remain activated during reperfusion (22).

In the heart, ischemic infarcts are marked by the death (both apoptotic and necrotic) of cardiomyocytes, as well as by fibrosis surrounding the site of vessel occlusion. In contrast, to expression of active MKK6 and MKK7, which foster hypertrophy, expression of active MKK3 mutants causes cardiomyocyte apoptosis (329, 330) in a cultured cardiomyocyte cell model. MKK6 activates all forms of p38, whereas MKK3 preferentially recruits p38α and p38β. These results suggest that the SAPKs, p38γ, and p38δ are critical for promoting cardiomyocyte hypertrophy, whereas p38α and p38β are more important for eliciting cardiomyocyte apoptosis (330).

The mechanisms by which reperfusion, as occurs in surgical bypass or balloon angioplasty, incurs cell injury remain largely enigmatic. It is generally accepted that reoxygenation of ischemic/atherosclerotic tissues results in the production of ROS, triggering oxidant stress-induced upregulation of inflammatory cell adhesion molecules such as the selectins and vascular cell adhesion molecule-1 (VCAM-1). The expression of adhesion molecules attracts neutrophils and macrophages, contributing to a local pathogenic inflammatory response which includes the release of inflammatory cytokines of the TNF family. Oxidant stress itself also upregulates TNF expression. Together, these effects are thought to contribute to cardiomyocyte apoptosis as well as to vascular restenosis, common unwanted side effects of bypass or angioplasty. Several recent studies have shown that E-selectin and VCAM-1 expression are AP-1 dependent (252), suggesting that the SAPKs, which are activated during reperfusion episodes, may initiate reperfusion injury. Inasmuch as ASK1 is activated both by oxidant stress, and by TNF, through an
oxidant stress-dependent mechanism, it will be important to ascertain if ASK1 can elicit cardiovascular injury.

In contrast, a recent study has demonstrated that MEKK1 suppresses oxidant stress-induced apoptosis in ES cell-derived cardiomyocytes (209). Thus mekk1 −/− ES cells were stimulated to differentiate into embryoid bodies from which cardiomyocytes were isolated and cultured. Disruption of mekk1 rendered these cardiomyocytes considerably more sensitive to the cytotoxic effects of oxidant stress (H2O2), increasing oxidant stress-induced apoptosis. Coincident with this, oxidant stress- and hypoxia-induced activation of SAPK was abrogated in the mekk −/− cells, indicating that MEKK1 negatively regulates the expression of TNF (209). This result is in contrast to results indicating that SAPKs are necessary for TNF release from macrophages stimulated with lipopolysaccharide (acting presumably through TLR4, see sect. vG). The basis for this difference is unclear, but, as is discussed above for developmental apoptosis in the brain (see sect. vC), this difference highlights the ability of the SAPKs to regulate apparently opposing biological processes depending on the cell type.

F. MEKK3 and Cardiovascular Development

Recent genetic studies suggest that stress-activated MAPK pathways are also critical not only to the response of cardiovascular cells to extracellular stress but to developmental cues that regulate cardiovascular development. Thus mice in which mekk3 is disrupted die in utero at embryonic day 11. In these animals there is a severe disruption of blood vessel development and angiogenesis. Interestingly, this occurs without a loss of expression of vascular endothelial cell growth factor or of its receptor (349). The development of embryonic but not maternal blood vessels in the placenta is particularly impaired in mekk3 −/− embryos, indicating an intrinsic defect in the endothelial cells (349). Inasmuch as MEKK3 was shown to activate MEF2C, a transcription factor critical to cardiovascular development, via the p38 pathway (349), it is plausible to speculate that MEKK3 regulation of p38 is especially important to embryonic angiogenesis.

G. The SAPKs Are Key Regulators of T-Cell Maturation, Activation, and Protection From FasL Apoptosis

1. T-cell receptor signaling and T-cell maturation

Three key gene disruption studies indicate a complex role for the SAPKs in T-cell maturation and function. Antigen presenting cells (APCs), such as macrophages and dendritic cells, present antigen to naïve T cells through the T-cell receptor (TCR), the CD3 subunit of which, in conjunction with CD28, recruits the TCR/CD28 costimulatory pathway. Activation of the costimulatory pathway culminates in the clonal proliferation and maturation of CD4+/CD8+ double positive cells to CD4+ TH cells and in the production of certain cytokines such as IL-2. While stimulation of either the TCR (CD3) or CD28 alone is insufficient for SAPK activation, TCR/CD28 costimulation results in strong SAPK and, hence, AP-1 activation. AP-1, in turn, is necessary for T-cell maturation and the development of acquired immunity (discussed below). IL-2 transcription is robustly activated by TCR/CD28 costimulation and is a particularly prominent target for the SAPKs (147, 293) inasmuch as the IL-2 promoter contains AP-1 and CRE elements (targets for the SAPKs and p38s) along with NF-κB and NF-AT sites.

Upon initial differentiation, CD4+ TH cells may then become either TH1 or TH2 effector cells. TH1 cells produce interferon-γ and promote the Ig class switching of B cells to those isotypes that engender phagocytosis by macrophages. Thus TH1 cells stimulate phagocytosis-dependent host responses and are important for the elimination of cells infected with viruses and other intracellular microbes (cell-mediated immunity). TH2 cells, on the other hand, produce IL-4 and IL-5. IL-4 induces B-cell class switching to IgE or, in humans, IgG4, neither of which can activate phagocytosis. IL-5 instead activates eosinophils. Thus TH2 cells contribute to phagocytosis-independent, IgE/eosinophil-mediated defenses (humoral immunity), such as those against helminth parasites.

Differentiation to TH1 or TH2 cells and, hence the type of immune response, are governed in part by the cytokine milieu to which the immature TH cells are exposed during antigen presentation. Thus, in addition to presenting antigen, some APCs (macrophages and dendritic cells in particular) produce IL-12. IL-12 promotes differentiation to TH1 cells (the type I cytokine immune response), while mature T cells (TH2 cells in particular, which can act as a second type of APC) produce IL-4, leading to further differentiation of TH1 to TH2 cells.

SAPKγ suppresses the differentiation of TH lymphocytes into TH2 cytokine-producing cells (76). Thus immature TH cells from mice in which sapkγ was disrupted were refractory to costimulatory SAPK activation, despite the continued presence of SAPKα. Thus in the TH1 stage, before differentiation into TH1/TH2 cells, SAPKγ is the only Jun kinase recruited by costimulation. Costimulation of the sapkγ knockout cells elicited indistinguishable levels of IL-2 production, suggesting that SAPK is not a rate-limiting pathway in TCR/CD28-stimulated IL-2 production (76). On the other hand, costimulation triggered enhanced proliferation in the knockout cells compared
with controls. TCR engagement alone did stimulate IFN-γ production, a hallmark of TH1 differentiation; however, CD28/TCR costimulation, which recruits SAPKα (293), failed to enhance IFN-γ production (76). In contrast, TCR engagement alone stimulated the production of exaggerated levels of the TH2 markers IL-4, IL-5, and IL-10. Thus deletion of sapkγ renders TH cells hyperresponsive to TCR engagement in the absence of costimulation and favors production of TH2 marker cytokines (76). As noted above, this response contributes to the maturation and differentiation of naive T cells into TH1 cells. Flavell and colleagues (187) deleted the mkk3 gene. These mice were viable and fertile but showed severely impaired macrophage and dendritic cell IL-12 production. In addition, these mice were defective in interferon-γ production, and antigen-driven differentiation of naive T cells was significantly reduced. Accordingly, deletion of mkk3 cripples the type I cytokine immune response (187). In cultured macrophage cells, pharmacological inhibition of the p38 pathway blocks IL-12 gene transcription, indicating that a significant component of p38’s effect on IL-12 expression is transcriptional.

SAPKα/JNK2 is apparently required for peripheral T-cell activation. Mice were generated wherein sapkα was deleted (261). The requirement for SAPKα differs from that for SAPKγ. Whereas deletion of sapkγ/jnk1 enhanced costimulatory proliferation and TCR-stimulated IL-4 production, while having no effect on TCR-stimulated IL-2 production, deletion of SAPKα reduced TCR-mediated proliferation and production of IL-2, IL-4, and interferon-γ markers of peripheral T-cell activation (76, 261). B-cell activation in the sapka knockout was unimpaired. In addition, CD3-induced apoptosis of immature (CD4+/CD8−) T cells was reduced in sapka−/− animals. Thus, in apparent contrast to SAPKγ, which suppresses T-cell differentiation, SAPKα is required for efficient peripheral T-cell activation (76, 261). MEKK2 has been implicated in APC-directed TCR signaling to MAPKs (p38s, and ERKs, but not SAPKs, see sect. μG2). It is unclear if MEKK2 is actually recruited by TCR alone or by costimulation (although the lack of SAPK activation would argue in favor of the former) (268). It will be important to determine if MEKK2 couples the TCR/CD28 costimulatory pathway to SAPKα.

2. SEK1 and protection from FasL apoptosis

Early in T- and B-lymphocyte development, ablation of autoreactive cells is mediated in part by FasL-stimulated apoptosis. Penninger and colleagues (226) disrupted sek1 in mice and observed that this deletion abrogated SAPK activation by anisomycin and heat shock in cultured ES cells (see sect. μF1). However, deletion of sek1 was early embryonic lethal. The lethality incurred upon deletion of sek1 is likely due to impaired hepatogenesis. Thus sek1−−/− embryos die early in embryogenesis, subsequent to the establishment of the primitive vasculature, coincident with early hepatogenesis. Lethality is accompanied by excessive apoptotic death in the liver, again indicating an antiapoptotic role for SEK1 (102, 227). To circumvent this problem, and examine the role of SEK1 (and, hence, the SAPKs) in immune cell function, rag2-deficient blastocysts were microinjected with sek1−−/− ES cells to produce chimeric mice in which a SEK1-deficient immune system developed against a wild-type background. T lymphocytes isolated from the chimeras were strikingly hypersensitive to FasL-induced apoptosis, suggesting that, consistent with the impaired hepatogenesis in sek1−−/− mice, SEK1 and its substrates actually protect T cells from FasL-induced apoptosis (102, 227).

H. The SAPKs and p38s Promote the Stabilization and Enhanced Translation of mRNAs Encoding Proinflammatory Proteins

Much of the preceding discussion of the mechanisms of SAPK and p38 signaling has focused on the role of these MAPK pathways in the phosphorylation of transcription factors and the regulation of gene transcription. However, as mentioned in section μE1, p38 can phosphorylate the MNKs, potentially activating their translational enhancement function. Several recent studies have highlighted roles for the SAPKs and p38s in the posttranscriptional and translational control of gene expression.

The signal-induced stabilization of mRNAs encoding proinflammatory proteins contributes to the robust and efficient induction of genes in the inflammatory response. Thus, proinflammatory stimuli such as bacterial lipopolysaccharide, IL-1, and TNF can trigger the stabilization of mRNAs encoding secondary cytokines (IL-6, IL-8) and prostaglandin biosynthetic enzymes [cyclooxygenase-2 (COX2)], and the disappearance of these mRNAs in the presence of the transcriptional inhibitor actinomycin D is substantially reduced in the presence of inflammatory cytokines or LPS. COX2 is an inducible isoform of prostaglandin H synthase and is the rate-limiting enzyme in extracellular signal-regulated inflammatory prostaglandin biosynthesis. Treatment of human monocytes with LPA, IL-1, or TNF stabilizes the COX2 mRNA in a reaction that is substan-
tially inhibited by SB203580. In these cells, at the doses of SB203580 used, p38 (presumably the α- and β-isoforms) and MAPKAP-K2 activity were significantly inhibited, and SAPK activity was unaffected. Thus it can be concluded that proinflammatory stimulation of COX2 mRNA stabilization is p38 dependent (67).

The mechanism by which p38s target mRNAs for stabilization has only begun to be elucidated. Rapidly turning over mRNAs such as those encoding COX2, interferon-γ, IL-1, IL-2, IL-3, IL-6, and IL-8 frequently contain AU-rich elements (AREs) in the 3′-untranslated region which are characterized by multiple copies of the sequence AUUUA. These elements, if transferred to stable mRNAs, such as that for β-globin, confer destabilization in resting cells and signal-induced stabilization. Winzen et al. (339) demonstrated that transient overexpression of active MEKK1 enhanced the stability of a chimeric mRNA containing β-globin and a 161 nt ARE from the IL-8 message. This reaction was not blocked by dominant interfering constructs of the SAPK, ERK, or NF-κB pathways. In contrast, expression of MKK6 enhanced mRNA stability, and MEKK1-induced stabilization could be reversed by a kinase-inactive, dominant inhibitory mutant of p38α (339). It can be inferred from these findings that the overexpressed MEKK1 was acting nonspecifically to recruit p38 which, in turn, triggered the stabilization of AU-rich mRNAs. Winzen et al. (339) also observed that a constitutively active form of MAPKAP-K2 could trigger mRNA stabilization through AREs, suggesting that p38-activated mRNA stabilization is mediated by MAPKAP-K2, a p38 substrate (95, 259, 339). The mechanism by which MAPKAP-K2 couples to ARE-mediated mRNA stabilization remains to be elucidated.

Despite the fact that AREs can regulate mRNA stability, this regulation is often differentially manifested. Thus T-cell activation stabilizes several ARE-containing cytokine mRNAs, including that for IL-2, while the mRNA for c-fos, which also contains an ARE, remains labile. This raises the possibility that additional elements can confer signal-induced mRNA stabilization. As mentioned above, induction of IL-2 expression is a hallmark of T-cell activation and requires costimulation mediated by the TCR (CD3) and CD28 (293). A significant component of IL-2 induction is at the transcriptional level; however, the signal-induced stabilization of IL-2 mRNA increases further the expression of IL-2 by activated T cells. The SAPKs were recently shown by Chen et al. (39) to regulate the stabilization of the IL-2 message through a cis-element spanning the 5′-UTR and the beginning of the coding region (39). Pretreatment with SB202190, a CSAILD similar to SB203580 that blocks p38 and, at high concentrations, SAPK, or cyclosporin, which blocks CD3/CD28 activation of SAPK (293) (and p38), prevented stabilization. However, the concentrations of SB202190 required to achieve inhibition of IL-2 mRNA stabilization were considerably higher than those necessary to completely inactivate p38, suggesting that the drug was affecting SAPK activity. In contrast, there was a close correspondence between the concentration of drug needed to reverse IL-2 message stabilization and to prevent in vivo c-Jun phosphorylation which is exclusively catalyzed by SAPKs (39). Consistent with a role for the SAPKs in mRNA stabilization, expression of constitutively active MKK7 or MEKK1, but not constitutively active MKK6 or Raf-1, triggered IL-2 message stabilization. Dominant inhibitory MKK7 blocked MEKK1- or CD3/CD28-stimulated IL-2 message stabilization (39).

The ability of lipopolysaccharide to trigger the acute phase response depends in large part on the transcriptional induction of TNF-α (signaled through the mammalian toll-like receptors, see sect. mE4), and enhanced TNF-α mRNA translation. Swantek et al. (296) used a TNF-α translational reporting system to demonstrate that a dominant inhibitory SAPK-β construct could block lipopolysaccharide-enhanced TNF translation (296). Interestingly, dexamethasone, a potent anti-inflammatory synthetic corticosteroid which has long been known to directly block AP-1 activity (147), could also strongly inhibit lipopolysaccharide activation of SAPK, but not activation of ERK or p38. Thus SAPKs are necessary not only for enhancement of TNF-α gene expression, but for increased translation of TNF-α (147).

VI. CONCLUDING REMARKS

During the past six years, our understanding of stress-activated signaling pathways has expanded dramatically. It has become apparent in the intervening time that these pathways can no longer be considered simple and linear. Instead, these mechanisms form complex signaling networks. At this time, the two most compelling issues in the study of stress-activated MAPK pathways are MAP3K → MEK → MAPK core pathway regulation and biological function.

A. Oligomerization, Adapter/G Protein Binding, Membrane Translocation, and Phosphorylation as Themes in MAP3K Regulation

Although a large number of potential MAP3K → MEK → MAPK core pathways, and their possible upstream activators and downstream targets have been identified, there is still a troubling lack of clarity concerning MAP3K regulation. In the case of stress-regulated MAP3Ks, this is due in large part to the high basal activity of these enzymes, making analysis of their activation difficult. It is becoming evident, however, that most MAP3Ks are regulated at least in part by four key processes: oligomerization, adapter protein binding, consequent membrane
translocation, and phosphorylation. Thus a role for oligomerization has been clearly established for Raf-1, ASK1, and MLK3. Moreover, MEKK1 selectively associates with oligomerized TRAF2, a process that could foster MEKK1 oligomerization-dependent activation. Whether this oligomerization triggers trans-autophosphorylation in a manner analogous to receptor Tyr kinases has not been determined. Alternatively, oligomerization could also render these kinases more attractive as targets for additional upstream activators.

There are some MAP3Ks for which putative upstream kinases have been identified (PAK3 for Raf-1, GCKs for MEKK1 and MLK3). Inasmuch as both the PAKs and, possibly, GCKs reversibly associate with membrane/receptor-associated polypeptides (Rho GTPases in the case of PAKs, TRAFs and SH3/SH3 adapters in the case of GCKs), the parallel recruitment of target MAP3Ks to membrane receptor complexes could enable the formation of MAP3K activating complexes.

G proteins and adapter proteins are clearly required for both the regulation of MAP3K oligomerization and activation by upstream kinases and may be a general paradigm of MAP3K regulation. The binding of TRAP oligomers to both MEKK1 and the group I GCKs, putative MEKK1 upstream kinases, is a potential example. Finally, MLK3 is activated, at least in part, by Cdc42-dependent oligomerization. HPK1, a putative upstream activator of MLK3, is activated by the SH2/SH3 adapter protein Crk/Crkl, which has also been implicated in the regulation of Rho family GTPases. Thus Tyr kinases might recruit MLKs by fostering Rho GTPase- and group I GCK-dependent activation. Clearly, it will be important to sort out the different roles of oligomerization, adapter protein binding, and upstream kinases in MAP3K regulation.

B. MAPK Pathway Biology

Genetic models were indispensable in the dissection of the Ras-ERK pathway. Although until recently there has been a comparative paucity of genetic models from which to draw conclusions as to stress-activated MAPK pathway regulation, new emerging genetic models such as the dorsal closure pathway in Drosophila, coupled with the completion of the C. elegans and other genome sequencing projects, should make it possible to understand the epistatic relationships between MAP3Ks and their upstream activators.

Placing these pathways in the context of human disease pathology will be more difficult. In this instance, pharmacological inhibitors such as PD98059 or SB203580, plus mouse knockout and transgenic approaches, will enable a greater understanding of how mammalian stress-activated MAPK pathways relate to disease and will identify important new drug targets.

The original draft of this article was completed in March 2000.

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