MAMMALIAN MAPK SIGNAL TRANSDUCTION PATHWAYS ACTIVATED BY STRESS AND INFLAMMATION: A 10-YEAR UPDATE

John M. Kyriakis and Joseph Avruch

I. INTRODUCTION

Mitogen-activated protein kinase (MAPK) signal transduction pathways are ubiquitous and highly evolutionarily conserved mechanisms of eukaryotic cell regulation. The multiple MAPK pathways present in all eukaryotic cells enable coordinated and integrated responses to diverse stimuli. These stimuli include hormones, growth factors, cytokine, agents acting through G protein-coupled receptors, transforming growth factor (TGF)-β-related agents that through Ser-Thr kinase receptors, pathogen-associated molecular patterns (PAMPs) and danger-associated molecular patterns (DAMPs) that recruit pattern recognition receptors (PRRs), and environmental stresses. MAPK pathways, once activated, exert major effects on cell physiology. Thus MAPKs orchestrate the recruitment of gene transcription, protein biosynthesis, cell cycle control, apoptosis, and differentiation (33, 152, 232, 235).

The three-tiered so-called “core signaling module” is a defining feature of MAPK pathways. Within this MAPKs are phosphorylated and activated via simultaneous Tyr and Thr phosphorylation within a distinct, and evolutionarily conserved Thr-X-Tyr motif in the kinase subdomain VIII activation loop. This phosphorylation is catalyzed by members of the dual-specificity MAPK kinases (MAP2Ks, also called MEKs or MKKs). The MAP2Ks are activated by Ser/Thr phosphorylation, again within a conserved motif in kinase subdomain VIII. This phosphorylation is catalyzed by a bewilderingly diverse group of protein kinase families referred to collectively as MAPK kinase kinases (MAP3Ks). MAPK core signaling modules are themselves regulated by a poorly understood array of upstream components (33, 152).

MAPKs are proline directed. Thus they phosphorylate Ser/Thr residues only if they are followed immediately by Pro residues. It is important to note, however, that proline directedness itself is insufficient to account for the remarkable substrate selectivity of the different MAPK subgroups. A notable property of the physiological substrates of the MAPKs is the presence of specific MAPK docking sites (MAP3Ks). MAPK core signaling modules are themselves recognized by comple-
mentary docking motifs on the cognate MAPKs. This confers a high degree of substrate specificity that would not be possible if proline directedness were the sole criterion for MAPK substrate recognition (15, 74, 136, 137, 138, 288, 289, 341).

In contrast to the high degree of MAPK substrate selectivity, individual elements within MAPK core signaling modules frequently function promiscuously in several pathways and are often subject to regulation by multiple stimuli (TABLE 1).

Despite the apparent ability of MAPK components to function in multiple pathways, the selectivity of MAPK pathways as well as their efficiency must be preserved. In this capacity, scaffold proteins function to sequester select MAPK pathway elements, thereby maintaining a critical degree of pathway integrity and signaling efficiency. In some cases, scaffold proteins are distinct polypeptides that bind specific MAPK pathway components. Alternatively, core MAPK signaling components themselves also possess intrinsic scaffolding properties (203).

II. THE ERK, JNK, AND P38 MAPK CORE PATHWAYS AND THEIR TARGETS: ACTIVATION BY ENVIRONMENTAL STRESSES AND INFLAMMATORY MEDIATORS

A. The ERKs: Not Just Effectors for Mitogenic Signaling

Mammals express multiple MAPK pathways. The majority of these are, along with the nuclear factor-κB (NF-κB) pathway, recruited by stress and inflammatory stimuli rather than by mitogens. Accordingly, these pathways represent a substantial trove of potentially important targets for novel anti-inflammatory drugs.

The extracellular signal-regulated kinases (ERKs-1 and -2; MAPKs-3 and -1, respectively) were the first mammalian MAPKs to be identified. The ERKs are most familiar as insulin and mitogen-activated MAPKs recruited by agonists that engage the Ras protooncoprotein. Ras, in turn, recruits the Raf family of MAP3Ks. The Rafs activate two ERK-specific MAP2Ks: MEK1 and MEK2 (MAP2Ks-1 and -2, respectively). The biochemistry, biology, and regulation of Ras-dependent ERKs activation have been extensively reviewed (192, 229).

In many instances, the ERKs can also be activated, in a manner independent of Ras, by proinflammatory stimuli including cytokines of the tumor necrosis factor (TNF) family, by PAMPs, such as lipopolysaccharide produced by invading microbial pathogens, and by DAMPs, endogenously produced danger signals such as oxidized low-density lipoprotein (LDL) in atherosclerosis and crystalline uric acid in gout. PAMPs/DAMPs engage PRRs [including those of the IL-1 receptor/toll-like receptor (TLR) family]. As is discussed below, these mechanisms of ERK activation play an unexpectedly important role in innate immunity and inflammation.

The ERKs are encoded by two genes, ERK1 and ERK2 (TABLE 1). As with most conventional MAPKs, the ERKs require dual Tyr and Thr phosphorylation at two closely spaced residues in the activation loop of subdomain VIII: Thr185-Glu-Tyr187 (ERK2) or Thr203-Glu-Tyr205 (ERK1) (229).

B. The c-Jun NH$_2$-Terminal Kinases, a MAPK Group Activated by Growth Factors, Environmental Stresses, and Proinflammatory Stimuli

The c-Jun NH$_2$-terminal kinases (JNKs) (TABLE 1) were initially identified as a protein kinase activity purified from cycloheximide-treated rat liver. The purified kinase was able to phosphorylate the c-Jun transcription factor at Ser63 and -73, sites key to the regulation of c-Jun and activator protein-1 (AP-1) transactivation function (245). Of note, the liver kinase phosphorylated c-Jun at a substantially higher rate than that observed for the ERKs. Independently, JNKs were identified as a Jun kinase activity that adhered to immobilized c-Jun (59, 163–165, 225, 320).

JNKs, while activated by mitogens, are also vigorously activated by a variety of environmental stresses (heat shock, ionizing radiation, oxidants), genotoxins (topoisomerase inhibitors and alkylating agents), ischemic reperfusion injury, mechanical stress, vasoactive peptides, proinflammatory cytokines, PAMPs/DAMPs and, as expected for a kinase activated in liver by cycloheximide, translational inhibitors (cycloheximide and anisomycin) (59, 165, 320).

The JNKs are also activated by tunicamycin. This is conceptually important inasmuch as tunicamycin, which inhibits N-linked protein glycosylation, causes the accumulation of misfolded proteins exclusively within the lumen of the endoplasmic reticulum (ER). This leads to ER stress, and indeed, tunicamycin serves as a model for ER stress induction. The ability of an ER stress to activate the cytosolic JNK (165) implicated the JNKs as important effectors for the ER stress response. As discussed in section IV, high-fat diet can trigger ER stress in vivo, and this may play a critical role in triggering insulin resistance and high-fat diet-induced inflammation.

The JNKs are encoded by three genes JNKs 1–3, also called MAPKs 8, 9, and 10, respectively (59, 163–165) (TABLE 1). Each JNK isoform contains the MAPK characteristic Thr-X-Tyr phosphoacceptor loop in kinase subdomain VIII. For JNKs, the sequence is Thr183-Pro-Tyr185. Each JNK gene is subject to differential hnRNA splicing, both within the
## Table 1  Nomenclature for mammalian MAPK pathway components

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<th>Name</th>
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<th>Substrates/Effectors</th>
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The catalytic domain at a region spanning subdomains IX and X (resulting in \( \text{H9251} \) and \( \text{H9252} \)-JNKs, respectively, TABLE 1), and at the extreme COOH terminus to produce 46- and 54-kDa polypeptides (respectively, types 1 and 2 JNKs, TABLE 1). Accordingly, 12 JNK polypeptides exist. The functional significance of the type 1 and type 2 isoforms is unclear. The \( \text{H9251} \) and \( \text{H9252} \) JNKs apparently differ modestly in their affinities for substrates (47, 59, 137, 165, 320).

### C. The p38 MAPKs Are Activated by Stresses and Proinflammatory Stimuli

The first p38 MAPK identified, the \( \alpha \) isofrom, was isolated on anti-phosphotyrosine immunoaffinity beads as a P-Tyr protein in extracts of endotoxin-treated cells. cDNA cloning revealed that p38\( \alpha \) was remarkably closely related to the \textit{Streptomyces cerevesiae} osmosensing MAPK \textit{HOG1}. Thus, as with Hog1p, the phosphoacceptor motif of the p38s has the sequence Thr-Gly-Tyr (104, 108). p38\( \alpha \) was also identified independently and contemporaneously as a stress- and IL-1-activated kinase that could phosphorylate and activate MAPK-activated protein kinase-activated protein kinase-1 (MK2; sect. II D1). MK2 is a member of a Ser/Thr kinase family that, among other substrates, phosphorylates and activates the small heat shock protein Hsp27 (80, 237).

There are four p38 genes (TABLE 1): p38\( \alpha \)-\( \delta \), also called MAPK-14 (p38\( \alpha \)), -11 (p38\( \beta \)), -12 (p38\( \gamma \)), and -13...
Contemporaneously, p38 was isolated as a target for a class of experimental pyridinyl-imidazole anti-inflammatory drugs, the most extensively characterized of which is the compound SB203580 (171). In vitro assays demonstrated that only p38 and p38 are inhibited by SB203580 and its relative compounds. p38 and p38 are not at all affected by these drugs in vitro or in situ (98). The reason for the selective inhibition of p38 and p38 by SB203580 is that Thr106 resides in the hinge of the ATP binding pocket of p38 (and p38). In this pocket, Thr106 interacts with a fluorine atom, the SB203580. This positions the drug such that it interacts with His107 and Leu108 of the ATP binding pocket-blocking ATP binding. Substitution of the more bulky amino acids found in p38 or p38 (Met, and Pro or Phe, respectively, in both cases) for Thr106 abolishes SB203580 binding. Conversely, if Thr, corresponding to Thr106, is used to replace the bulky amino acids of p38, p38 (or even of JNK1), the resulting mutants are rendered at least partially sensitive to SB203580 (42, 75, 110, 246).

D. Key Substrates of the ERKs, JNKs, and p38s

The ERKs, JNKs, and p38s phosphorylate both other protein kinases and transcription factors, highlighting the importance of these MAPKs to inflammatory and stress responses.

1. Protein kinases

A) RIBOSOMAL S6 KINASES (RSKS), SELECTIVE SUBSTRATES FOR THE ERKS. RSKs are a family of Ser/Thr kinases that contain two tandem protein kinase domains: the COOH-terminal kinase domain is in the calcium-calmodulin kinase subfamily, and the NH2-terminal kinase domain is in the AGC subfamily. RSKs are so named for their phosphorylation of the small ribosomal protein S6, in *Xenopus* oocytes. However, RSKs do not represent the major somatic cell S6 kinases (these are the S6Ks). The RSKs were among the first MAPK substrates identified, and demonstration of ERK activation of RSK was a major breakthrough in the elucidation of MAPK pathways. JNKs and p38s do not regulate the RSKs. RSKs are activated through the combined activity of ERKs and 3-phosphoinositide-dependent kinase-1 (PDK1). ERKs activate the COOH-terminal catalytic domain by phosphorylating a Ser/Thr residue in the activation loop (e.g., Ser573 in human Rsk1) and participate in the activation of the NH2-terminal catalytic domain by phosphorylating a Ser-Pro site (e.g., Ser363 in human Rsk1) in the “turn” motif beyond the catalytic domain of the NH2-terminal kinase. The activated COOH-terminal catalytic domain then phosphorylates Ser380 (in a Phe-Ser-Phe motif) located further downstream of the NH2-terminal kinase domain. This provides a docking site for PDK1, which phosphorylates Rsk1 at Ser222 in the activation loop of the NH2-terminal catalytic domain, a final phosphorylation event required for full activation of Rsk. It is the NH2-terminal kinase domain that is likely responsible for phosphorylation of Rsk substrates. (FIGURE 1). Phosphorylation of Rsk substrates is likely mediated by the Rsk the NH2-terminal kinase domain (49, 231).

The RSKs can also be activated, selectively in dendritic cells and possibly macrophages, through an alternative mechanism. Here, TLRs trigger activation of p38 which, in turn, stimulates the activities of MKs-2 and -3 (see below). Genetic and biochemical evidence shows that these kinases phosphorylate Ser380 (human Rsk1), which is phosphorylated in cells other than dendritic cells by the ERK-activated COOH-terminal catalytic domain. Thus this MK2/3-mediated phosphorylation bypasses the necessity for phosphorylation of Rsk at the COOH-terminal kinase domain (Ser573). However, ERK phosphorylation of Ser363 in the

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**FIGURE 1.** MAPK regulation of downstream protein kinases.
NH₂-terminal domain is still required, and indeed, both ERK and p38 MK2/3 are necessary for optimal TLR activation of dendritic cell Rsk. In this dendritic cell system, MK2/3-catalyzed phosphorylation of Rsk Ser380 still provides the PKD1 recognition site which facilitates Ser222 phosphorylation (348).

This alternative mechanism apparently functions selectively in dendritic cells (although it may also function in macrophages) and is necessary for TLR-stimulated proinflammatory dendritic cell phagocytosis and pinocytosis of antigens, as well as for later induction of IL-10 and for resolution of inflammation (348) (see sect. IV.C).

B) MITOGEN-ACTIVATED PROTEIN KINASE-ACTIVATED PROTEIN KINASES (MK)-2, -3, AND -5. MAPK-activated kinase-2 (MK2) the structurally related MK3 and MK5 (also called p38-regulated and activated kinase, PRAK) are a small family of Ser/Thr kinases strongly activated by stresses, proinflammatory cytokines, and PAMPs/DAMPs. MK2/3 both consist of an NH₂-terminal regulatory domain and a COOH-terminal kinase domain. They are unrelated to the Rsk. Recombinant MK-2, -3, and -5 phosphorylate the small heat shock protein Hsp27 in vitro (123, 168, 193, 205, 269, 275, 276). However, this substrate promiscuity may not occur in situ inasmuch as only endogenous MK2 can robustly phosphorylate Hsp27, while MK3 does so much more weakly. Endogenous MK5 cannot phosphorylate Hsp27 at all. MK2 can also phosphorylate a number of mRNA binding proteins implicated in the signal-induced, posttranscriptional stabilization of mRNAs, notably those encoding proinflammatory cytokines (87, 233, 234, 265). This is discussed in detail in section IV.C5.

When not phosphorylated, Hsp27 aggregates into high-molecular-weight multimers. It is these complexes that serve as molecular chaperones. Hsp27 phosphorylation, catalyzed by MK2/3 at Ser15, Ser78, Ser82, and Ser90, coincides with the dissociation of Hsp27 into monomers and dimers and redistribution of Hsp27 to the actin cytoskeleton. Hsp27 redistribution may lead to the reorganization of F-actin into stress fibers, thus affecting cell motility (123, 168).

As is discussed in section IV.C5, MK2 can also phosphorylate a number of polypeptides that bind to mRNAs and modulate their stability. This process enables p38, through MK2, to regulate posttranscriptionally the in vivo and in situ expression of several proinflammatory cytokine genes including that for TNF (57, 87, 185, 228, 233, 234, 238, 265).

Finally, as noted above, MK2 and MK3 are required for optimal activation, by TLRs, of dendritic cell Rsk and for consequent dendritic cell phagocytic function (348).

MK2 is phosphorylated at Thr24, Thr222, and Ser272 and activated by p38α and p38β (but not by p38γ or p38δ). In line with the regulation of MK-2, -3, and -5 as well as Hsp27 by p38α and p38β, these processes are inhibited by pyridyl-imidazole p38α/β inhibitors such as SB203580 (14, 123, 168, 193).

C) MAPK-INTERACTING KINASES. A site of translational regulation for many mRNAs is the the N7-methylguanosine 5’ cap structure. Eukaryotic initiation factor (eIF)-4E (eIF-4E), an N7-methylguanosine-binding protein, tethers mRNAs onto eIF-R, a scaffold protein, which also binds eIF-4A, an RNA helicase. The eIF-4A polypeptide, together with eIF-4B, an RNA binding protein, unwinds secondary structure in the mRNA 5’ untranslated region. This facilitates the scanning of the mRNA by the 40S ribosomal complex which moves along the mRNA to the ATG translational initiation site. The complex of eIF-4A, -B, -G, and -E is referred to collectively as eIF-4F (272).

The translational repressor protein 4E-binding protein-1 (4E-BP1 also referred to as phosphorylated heat- and acid-stable protein regulated by insulin, PHAS-I) negatively regulates the eIF-4E-eIF-4G interaction. Insulin and mitogen-stimulated phosphorylation of 4E-BP1, catalyzed by mammalian target of rapamycin (mTOR), results in the dissociation of 4E-BP1 from eIF-4E and the release of eIF-4E. As a consequence, eIF-4E is incorporated into the eIF-4F complex, thereby enabling translational initiation (272).

Insulin, mitogens, and environmental stresses also stimulate the regulatory phosphorylation of eIF-4E at Ser209. Evidence has been presented indicating that this phosphorylation contributes to eIF-4E’s oncogenic potential (86, 297, 318), but its specific role in translational regulation remains uncertain (21, 272).

The likely eIF-4E Ser209 kinases are two closely related Ser/Thr kinases MAPK interacting kinases (MNKs)-1 and -2. MNKs, as their names suggest, interact with and are in vitro and in vivo substrates of MAPKs. Both ERKs 1/2 (in response to insulin and mitogens) and by the p38s (in response to cytokines and stress) can catalyze phosphorylation and activation of the MNKs (85, 311, 312), indicating that, as with AP-1 (see below), MNKs are a site of integration of stress and mitogen signaling pathways. Given their role in modulating eIF4E phosphorylation, which has been linked to tumorigenesis (86, 297, 318), it is reasonable to suggest that MNKs promote tumor development (FIGURE 1).

D) MSK1/2. Mitogen- and stress-activated protein kinases (MSK)-1 and -2 are a family of Ser/Thr protein kinases that, like the Rsk, possess two tandem protein kinase domains. The activity of MSK1 is activated by both the ERKs and p38 MAPKs (56).
The bZIP transcription factor cAMP response element binding protein (CREB) binds and trans activates genes containing the cAMP response element (CRE). Numerous divergent stimuli including mitogens, stresses and, of course, agonists that elevate cAMP (via PKA), can activate CREB trans activating function, a process that correlates with phosphorylation of CREB at Ser133. In this context, CREB serves as a point of signaling convergence, responsive to multiple inputs in different physiological contexts. Thus several protein kinases including the Rsk (333) and MK2 (285) are capable of phosphorylating CREB Ser133 in response to mitogen or stress. In addition, substantial evidence indicates that MSK1 is a physiologically relevant stress- and mitogen-activated CREB Ser133 kinase (FIGURE 1). Regulation of CREB by PKA has been associated with learning and memory (191). In contrast, in vivo, MSKs, possibly through CREB regulation, may act redundantly as negative regulators of innate immunity (6, 56) (section IVC1).

2. Transcription factors

A) CHOP/GADD153. The bZIP, CCAAT enhancer binding protein (c/EBP) family transcription factor C/EBP homologous protein (CHOP), also called growth arrest and DNA damage-155 (GADD153) (214), is induced at the transcriptional level and functionally activated in response to genotoxic and inflammatory stimuli. The transcriptional regulatory functions of CHOP/GADD153 are activated by stimulus-induced phosphorylation of Ser78 and Ser81. CHOP/GADD153 acts as both a transcriptional repressor (of certain cAMP-regulated genes) and a transcriptional activator (of a subset stress-induced genes). Genotoxin-induced cell cycle arrest at G$_1$/S is mediated in part by CHOP/GADD153. This is an important process, as cell cycle arrest permits DNA repair prior to S phase, thereby preserving the integrity of the genome. Inasmuch as p38$_\alpha$, but not JNK or ERK, phosphorylates CHOP/GADD153 at Ser78 and Ser81 in vivo and in vitro, it is likely that p38$_\alpha$ is a stress-activated regulator of CHOP/GADD153 function (307) (FIGURE 2).

B) NFAT. The nuclear factor of activated T cells (NFAT) family of transcription factors is distantly related to Rel/NF-kB. In resting cells, NFATs are phosphorylated by casein kinase-Ia and, possibly, glycogen synthase kinase (GSK)-3 at five or six sites. This masks the NFAT nuclear localization signal resulting in the retention of NFATs in the cytosol. Stimulus-dependent Ca$^{2+}$ entry activates the Ca$^{2+}$-dependent phosphatase calcineurin (phosphatase 2B). Calcineurin then dephosphorylates NFATs, unmasking NFAT’s nuclear localization signal thereby enabling NFAT nuclear translocation. NFAT DNA binding affinity is also enhanced by dephosphorylation. NFAT and AP-1 sites are often closely apposed in many promoters. This favors the cooperative binding and synergistic trans activation of numerous genes (IL-2, IL-4, IL-5, and CD40L are examples) (113, 184). The immunosuppressants FK506 and cyclosporin-A are potent calcineurin inhibitors, which accounts for essentially all of their biological activity (256).

The Ca$^{2+}$-mediated nuclear translocation of NFATs is strongly inhibited by serum factors. Neither casein kinase nor GSK3 is serum-stimulated. Accordingly, it was suggested that serum-dependent inhibition of Ca$^{2+}$-mediated NFAT translocation was mediated by serum-responsive kinase cascades. Inasmuch as nuclear translocation is essential to NFAT function, these kinases are considered NFAT inhibitors (36, 113). In line with this, the JNKs phosphorylate NFAT4 (also called NFATc3) at Ser163 and Ser165, coinciding with an inhibition of stimulus-induced NFAT4 nuclear translocation (36) (FIGURE 2A).

Coadministration of phorbol myristate acetate (PMA) and ionomycin stimulates JNK-dependent phosphorylation of T lymphocyte NFAT2 (also called NFATc1) at Ser117 and -172. As with NFAT4, this phosphorylation inhibits or delays the accumulation of NFAT2 in the nucleus. This inhibition is due to the ability of JNK phosphorylation of NFAT2 to prevent the binding of calcineurin, an obligate step in NFAT2 activation (37). NFAT2 activity is critical to the polarization of T$_{H}$ cells to the T$_{H}$2 effector phenotype, consistent with the idea that JNK activation biases T$_{H}$ polarization to the T$_{H}$1 trajectory at least in part through NFAT2 inhibition. In line with this, disruption of JNK1 or both JNK1 and -2 leads to the preferential accumulation of T$_{H}$2 cells (37, 66, 67) (see sect. IVC2).

C) AP-1. The JNKs and p38s are the major Ser/Thr kinases responsible for the recruitment of the heterodimeric transcription factor activator protein-1 (AP-1) (147, 166); however, the ERKs also function to regulate AP-1 (FIGURE 2, A AND C). AP-1 dimers consist of bZIP transcription factors of the Jun group, primarily c-Jun and JunD, paired either with members of the fos (usually c-Fos) or activating transcription factor (ATF, usually ATF2) families. Characteristics of bZIP transcription factors are leucine zippers that enable homo- and heterodimerization AP-1 components are most commonly observed as Jun-Jun, Jun-Fos, or Jun-ATF dimers (143, 260, 300, 301).

AP-1, via Jun family components, binds to the tetradeacetylphorbol acetate (TPA) response element (TRE). Insofar as ATFs, including ATF2, in the CREB subfamily of bZIP transcription factors, AP-1 heterodimers containing ATFs can bind both to the TRE and to the CRE (113, 147). AP-1 is a pivotal trans activator of a number of genes recruited by stress and inflammation. These include the genes for IL-1 and IL-2, CD40, CD30, TNF, and c-Jun itself. AP-1 also participates in the transcriptional induction of proteases and cell adhesion proteins, such as E-selectin, which are important to inflammation (143, 260).
Regulation of AP-1 is complex and occurs at several levels. These include the direct phosphorylation/dephosphorylation of AP-1 components, the stabilization of AP-1 components (notably c-Jun), and the recruitment (typically via phosphorylation) of transcription factors that induce \( c\text{-}jun \) or \( c\text{-}fos \). These events can be activated independently by several signaling pathways (FIGURE 2).

Phosphorylation of c-Jun, in resting cells, at a region immediately upstream of the COOH-terminal of the DNA binding domain (Thr231, Thr239, Ser243, Ser249) inhibits DNA binding. This phosphorylation is catalyzed in situ by either glycogen synthase kinase-3 (GSK3) or casein kinase II (CKII), both of which are constitutively active in resting cells. Of note, GSK3 is phosphorylated and inactivated by a mechanism dependent on phosphatidylinositol-3-kinase (PI-3-K) (38). The same conditions that activate AP-1 foster GSK3 inhibition. Thus, under these circumstances, the c-Jun COOH-terminal phosphates are removed (17).

The \textit{trans} activating activity of c-Jun and ATF2 correlates strongly with phosphorylation within their NH\textsubscript{2}-terminal \textit{trans} activation domains (Ser63 and -73 for c-Jun, Thr69 and -71 for ATF2). The JNKs are the principal kinases responsible for c-Jun Ser63/73 phosphorylation (59, 165, 225, 260). Thus all stress- and TNF-activated c-Jun Ser63/73 kinase activity can be effectively removed either by immunodepletion or genetic disruption of JNK (165, 300, 301) (FIGURE 2A). JNK also phosphorylates JunD, at Ser90 and Ser100, a region of the JunD \textit{trans} activation domain similar to the phosphoacceptor domain of c-Jun (138).

JNK activity also regulates c-Jun stability, albeit in a complex and incompletely understood manner (FIGS. 2B and C).
c-Jun is susceptible to Lys48-linked ubiquitination and proteasomal degradation. Early studies (83, 204) indicated that the binding of JNK to c-Jun (which promotes c-Jun phosphorylation) targeted c-Jun for ubiquitination and degradation (by an unknown mechanism), whereas dephosphorylation of c-Jun Ser63/73 rendered c-Jun resistant to ubiquitination and degradation, prolonging its half life (FIGURE 2).

Consistent with a role for JNK in destabilization of c-Jun, JNKs can also indirectly contribute to ubiquitin (Ub)-dependent c-Jun degradation through phosphorylation and activation of the HECT domain E3 ubiquitin ligase Itch. Itch, so activated, then coordinates the E1 and Ubc7 (E2)-dependent ubiquitination of c-Jun (92). The MAP3K MEKK1 may regulate JNK-dependent Itch phosphorylation and, independently, may directly regulate Itch (73, 302).

JNKs and p38s phosphorylation of ATF2 at Thr69 and Ser71 in the trans activation domain correlates with activation of ATF2 trans activating activity (102). Whether the JNKs or p38s represent the dominant ATF2 kinases is cell and stimulus dependent (45, 201).

AP-1 activation also involves the induction of genes encoding AP-1 components, a process that can be activated by the ERKs, JNKs, and p38s (105, 143, 260) (FIGURE 3). Induction of c-fos, one of the most rapid transcriptional events, requires a cis-acting element in the c-fos promoter, the serum response element (SRE). A heterodimeric transcription factor containing two polypeptides, the serum response factor (SRF) and a member of the ternary complex factor (TCF) family, bind to the SRE and mediate c-fos induction (295).

TCF phosphorylation is central to regulation of this process. The TCF family includes Elk-1 and Sap-1a (295). The Elk1 COOH terminus (Ser383, Ser389) is phosphorylated by the JNKs and ERKs (but not p38). The corresponding residues (Ser381 and Ser387) on Sap1a are phosphorylated by the p38s (96, 131, 187, 322, 341). TCF phosphorylation enhances heteromerization with SRF and consequent trans activation at the SRE (FIGURE 2).

As their name suggests, transcription factors of the myocyte enhancer factor-2 (MEF2) subgroup of the MCM1-agamous and deficiens-SRF (MADS) box transcription factor family regulate genes involved in myocyte differentiation. However, some MEF2s, notably MEF2C, are expressed more widely and regulate diverse transcriptional events. Of the MEF2 family, only MEF2A and -C are MAPK substrates, a process that enhances MEF2A/C trans activating activity (349). MEF2A is phosphorylated at Thr312 and Thr319 by p38a (349). The p38s phosphorylate Thr293 and Thr300 of MEF2C (105). A MEF2C cis element is present in the c-jun promoter. Indeed, MEF2A or -C, once activated by p38s, can trans activate the c-Jun promoter. Accordingly, p38 activation can contribute to the induction of c-jun expression (105), which, in turn, contributes to the activation of AP-1 (105, 349).

3. MAPK substrates have specific MAPK docking sites which confer substrate for the JNKs and p38s and which interact selectively with distinct substrate binding motifs present on MAPKs.

JNK-catalyzed phosphorylation of c-Jun is an excellent example of the mechanism of substrate recognition by members of the MAPK family. Despite being “proline-directed” phosphorylating Ser/Thr residues only if followed immediately by proline-MAPK substrate specificity is directed in large part by the presence of binding sites on MAPK substrates. These binding motifs are often quite distant from the “proline-directed” phosphoacceptor sites and are typically specific for distinct MAPK subgroups. This enables the selective interaction between MAPKs and their physiological substrates (15, 288, 289) (TABLES 2 and 3).

There are two broad classes of MAPK binding site present on MAPK substrates. One, referred to as a D-domain, generally consists of a hydrophobic cluster of amino acid residues. These motifs are present in many transcription factor substrates of MAPKs. For example, the JNKs, but not the ERKs or p38s, bind c-Jun quite strongly through an interaction that requires a domain on c-Jun between residues 32 and 52, substantially distant from the phosphoacceptor sites Ser63 and Ser73. This JNK docking site overlaps with the so-called δ-domain (AAs 30–57), which has been linked to the regulation of c-Jun oncogenicity and is deleted in

![FIGURE 3. Regulation of MAPKs by MEK-dependent and -independent phosphorylation. All three MAPK groups listed are regulated by direct MEK phosphorylation; however, T cell p38α is also regulated by Lck/Zap70-mediated Tyr phosphorylation, which triggers auto-phosphorylation at the activating phosphoacceptor sites.](image-url)
oncogenic v-Jun (not surprisingly, v-Jun does not bind JNK and is not a JNK substrate) (47, 137, 138) (TABLES 2 and 3).

As with the c-Jun δ-domain, ATF2 contains a hydrophobic substrate binding motif (AAs 20–60) that lies well NH2 terminal to the phosphoacceptor sites (Thr69 and Thr71) (102). Likewise, Elk-1 has a MAPK docking site, the D-domain (AAs 312–335), that also resides significantly NH2 terminal to the phosphoacceptor sites (Ser383, Ser389) and is required for efficient ERK and JNK binding and phosphorylation. The D-domain is a common motif present on many MAPK interactors (289, 322, 341).

A second class of MAPK binding domain is comprised of a small stretch of basic amino acid residues. This domain is prevalent among protein kinase substrates of MAPKs (Rsk2, MNK1, and MKs-2–5) as well as MAPK regulators [notably the JNK-specific MAP2Ks MAPK kinase (MKK)-4 and some MKK7 isoforms, p38 MAP2Ks MKK3 and MKK6; as well as MAPK phosphatases (TABLES 2 and 3)]. This polybasic domain binds to the so-called common docking (CD) domain, an extracatalytic region found in all MAPKs. CD motifs are rich in acidic amino acid residues. The basic residues in these MAPK binding sites likely interact electrostatically with the cognate MAPK CD motifs, with differences among the basic MAPK docking sites and MAPK CD domains likely conferring a high degree of specificity among MAPKs for their substrates and activators (15, 288, 289).

### Table 2  MAPK and MAPK substrate/regulator docking sites

<table>
<thead>
<tr>
<th>MAPK Substrate</th>
<th>Hydrophobic Domain</th>
<th>Polybasic Docking Site</th>
</tr>
</thead>
<tbody>
<tr>
<td>c-Jun</td>
<td>25-AYGYSNPKILGSMYTLADP VGSLK-50</td>
<td></td>
</tr>
<tr>
<td>JunD</td>
<td>43-APPTSSMLKKDLALTSLADEGAAGLKLKKGL-68</td>
<td></td>
</tr>
<tr>
<td>JunB</td>
<td>26-SLSLHDKLLKPATTALNLADP YRGLK-51</td>
<td></td>
</tr>
<tr>
<td>MKK6</td>
<td>4-SKGKKKPNGLKIP-16</td>
<td></td>
</tr>
<tr>
<td>MNK1</td>
<td>399-CKSRARGGRLAAGAQAG-413</td>
<td></td>
</tr>
</tbody>
</table>

Hydrophobic and polybasic MAPK docking sites on representative MAPK substrates. For the Jun family members, only c-Jun and JunB can actually associate with JNKs. Only c-Jun and JunD, however, have phosphoacceptor sites. Thus JunD must heterodimerize with JunB or c-Jun to serve as JNK substrate. The site in c-Jun is associated with the δ-domain, see text for details.

E. Direct Activation of the ERKs, JNKs, and p38s by Specific MAP2Ks

1. Activation of the ERKs by MAP2Ks-1 and -2

As with most MAPKs, the ERKs are activated by dual Tyr/Thr phosphorylation within the activation loop (Thr185/Tyr187 for ERK2 within the characteristic motif Thr-Glu-Tyr). The existence of a MAPK “activator” was first demonstrated by fractionating cytosolic extracts of EGF-treated cells on ion exchange columns and assaying the fractions for an activity that could activate ERK1 and/or ERK2 in vitro. Purification and molecular cloning of this activity revealed a pair of dual-specificity (Thr/Tyr) protein kinases termed variously MAPK-kinases-1 and -2 (MAPKKs-1/2) or MAPK/ERK kinases-1 or -2 (MAP2Ks-1/2) (TABLE 1 and FIGURE 3) that could phosphorylate the ERKs at the activating phosphoacceptor sites (229).

MEK1 can be inhibited by two relatively specific pharmacologic agents: PD98059 and U0126. PD98059 prevents MEK1 phosphorylation by upstream kinases. U0126 does not prevent phosphorylation of MEK1 by upstream activators, but instead restrains MEK1 in an inactive conformation, preventing its signaling to downstream elements. Both of these compounds have been used extensively to identify pathways in which the ERKs play a dominant role (8, 9, 54).

2. JNK activation by MKK4 and MKK7

Activating JNKs phosphorylation occurs at Thr183 and Tyr185 (phosphoacceptor sites separated by a Pro residue) and is catalyzed by two MAP2Ks. MAPK-kinase-4 (MKK4, also called MAP2K4, SAPK/ERK kinase-1, SEK1; MKK4; JNK kinase-1, JNKK1; and SAPK-kinase-1, SKK1, TABLE 1) can phosphorylate and activate all three JNK isoforms in vivo and in vitro. MKK4 can also phosphorylate and activate p38 (60, 252) (FIGURE 3). MKK7 (also called MAP2K7, MEK7, JNKK2, and SKK4, TABLE 1) bears significant homology to the JNK-specific Drosophila MAP2K
hemipterous. MKK7 is expressed from several alternative hnrRNA splicing isoforms, each of which displays a strong preference for JNK, activating all isoforms equally well. MKK7 does not activate p38 at all (97, 182, 293, 329, 343) (FIGURE 3).

3. p38 activation by MKK3 and MKK6

The activating phosphoacceptor sites on the p38s are separated by a glycine residue (Thr180/Tyr182 for p38α). MKK3 (also called MAP2K3, MEK3, and SKK2) and MKK6 (also called MAP2K6, MEK6, and SKK3, TABLE 1) are both highly selective activators of the p38s and do not activate JNK or ERK under all conditions tested (FIGURE 3). There are very slight differences in the substrate specificity of MKK3 and MKK6. MKK3 preferentially activates p38α and p38β, while MKK6 can activate strongly all known p38 isoforms (44, 45, 60, 227).

4. MKK3/6-independent “noncanonical” activation of p38

p38α can also undergo MKK-independent activation. Two mechanisms have been documented. For the first, p38α directly interacts with the adapter protein TGF-β-activated kinase-1-binding protein-1 (TAB1; see sect. IIID4) leading to autophosphorylation at the classical Thr180/Tyr182 activating phosphoacceptor sites (FIGURE 3). This interaction, detected using overexpressed proteins, can be stimulated by another adapter protein, TNF receptor-associated factor-6 (TRAF6; discussed below, see sect. IIID), and may couple p38α to PAMP agonists (93). However, the overall physiological relevance of this mechanism is unclear. Indeed, mouse embryo fibroblasts (MEFs in contrast to the situation described below for T lymphocytes), in which both mkk3 and mkk6 are disrupted, show a profound defect in all known mechanisms of p38α activation. The exceptions are a small portion of the activation of p38α by hyperosmolarity, anisomycin, and peroxynitrite (142).

A second noncanonical p38α activation mechanism occurs exclusively in T lymphocytes and involves direct Tyr phosphorylation at a site outside of the classical Thr180/Tyr182 activating phosphoacceptor sites. This Tyr phosphorylation occurs at Tyr323 and is dependent on the nonreceptor Tyr kinase Lck and its effector Tyr kinase, Zap70 (FIGURE 3). Zap70 directly phosphorylates p38α at Tyr323. Tyr323 phosphorylation triggers p38α autophosphorylation at Thr180/Tyr182. Tyr323 lies in a linker region between the two lobes of the p38α polypeptide, a region implicated in homodimerization. It is possible that phosphorylation of this residue may promote trans phosphorylation or some other mechanism of activation (250, 251). Lck and Zap70 are pivotal to T cell receptor (TCR) signaling. As discussed in section IV C3, genetic studies document that this activation mechanism is essential to T cell function (135).

F. A Divergent Array of Proinflammatory and Stress-Activated MAP3K Families Are Upstream of the ERKs, JNKs, and p38s

1. General considerations

The stress-activated MAP2Ks, as with all MAP2Ks, are regulated by Ser/Thr phosphorylation. The regulatory phosphoacceptor sites lie within a conserved region the P-activation loop of subdomain VIII of the kinase domain. The (human) MEK1 phosphoacceptor sites are Ser218 and Ser222; those for MEK2 are Ser222 and Ser226. The human MK4 phosphorylation sites are Ser257 and Thr261, those for MKK7 are Ser206 and Thr210, those for MKK3 are Ser189 and Thr193, and those for MKK6 are Ser207 and Thr211 (44, 60, 71, 182, 201, 227, 229, 252, 293, 329, 343).

Consistent with the multiple different stimuli that recruit stress-activated MAPK pathways, a dauntingly diverse number of Ser/Thr kinases regulates stress-activated MAPKs, and the enzymology of these MAP3Ks is accordingly complex.

2. The MEKK group

The mammalian MEKKs consist of MEKK-1 and -4. NF-κB-inducing kinase (NIK, not to be confused with the germinal center kinase family member Nck interacting kinase, also abbreviated NIK, which is discussed below) is an additional MEKK. However, NIK is a specific activator of the NF-κB pathway (186) and will not be discussed here. The MEKK catalytic domains are, of mammalian kinases, most closely homologous to that of S. cerevisiae STE11, a MAP3K recruited both by mating pheromone and hyperosmolarity (108). Outside of the kinase domains, however, the MEKKs are structurally quite dissimilar. Mammalian MEKKs can, in some instances, catalyze the activation of multiple different MAP2Ks and interact with numerous putative regulatory proteins (16, 71, 95, 108, 169, 283, 335) (TABLE 1).

A) MEKK1, A RELATIVELY SELECTIVE ACTIVATOR OF THE JNK PATHWAY. MEKK1 (MAP3K1) is a very large (~190 kDa) polypeptide consisting of a COOH-terminal kinase domain [amino acids (AAs) 1221-1493] preceded by an extensive NH2-terminal domain (AAs 1–1221). The NH2-terminal domain includes two proline-rich segments (AAs 74–149 and 233–291) with putative binding sites for proteins with SH3 domains, a consensus binding site for 14–3-3 proteins (AAs 239–243), two PH domains (AAs 439–455 and 643–750), and two sites for cleavage by cysteine proteases of the caspase family (Asp871, Asp874) (22, 77, 169, 335).

The NH2 terminus of MEKK1 also contains a plant homeodomain (PHD)/really interesting new gene (RING) do-
main with intrinsic E3 ubiquitin (Ub) ligase activity (AAs 438–486). This activity assembles Lys48-linked polyubiquitin chains exclusively and can promote autoubiquitination as well as the ubiquitination of ERK2, a reaction which may attenuate Ras-dependent ERK activation and JNK activation (182). MEKK1 can also directly ubiquitinate and degrade c-Jun, in vitro and, apparently, in situ, in a manner dependent on its intact PHD/RING domain (MEKK1 disruption or mutagenesis of the PHD/RING domain Cys433 to Ala abolishes c-Jun ubiquitination) (331). This apparent direct MEKK1-dependent c-Jun degradation is linked to stress-induced apoptosis. Notably, MEKK1 ubiquitination of c-Jun occurred independently of prior c-Jun Ser63/73 phosphorylation, in contrast to earlier studies (83, 204) in which c-Jun ubiquitination/degradation correlated with c-Jun phosphorylation. However, as discussed below and in section IID2, the MEKK1 PHD/RING domain also binds the E3 Ub ligase Itch. Accordingly, it is somewhat unclear if MEKK1 directly or indirectly ubiquitinates c-Jun.

Thus MEKK1, in a manner dependent on the MEKK1 PHD/RING domain and phosphorylation of the MEKK1 polypeptide in its activation loop (Thr1381), can associate with the E3 ligase Itch, resulting in activation of Itch. This association occurs apparently independently of JNK (which can also activate Itch, Ref. 92; see sect. IID2) and of any discernable MEKK1-catalyzed phosphorylation of Itch (73). This interaction is necessary for TCR induction of IL-4 and IL-6. Evidence suggests that in vivo MEKK1-dependent ubiquitination and degradation of JunB impairs IL-4 production and consequent T_{h}2 differentiation. Thus MEKK1 negatively regulates T_{h}2 cytokine production (73). In contrast, Itch, downstream of MEKK1, and/or JNK may also trigger Itch-dependent c-Jun ubiquitination in vivo, contributing to T_{h}2 tolerance and reduced airway inflammation (302) (FIGURE 4C). It should be noted that these studies of T-cell polarization employed a MEKK1 knockout strategy that deleted solely the MEKK1 catalytic domain, leaving the remainder of MEKK1 intact. The resulting expression of MEKK1 fragments could lead to off target effects.

MEKK1 also contains a SWI2/SNF2 and MuDr (SWIM) domain (AAs 333–361, FIGURE 4). The function of this domain is unclear. However, there is some indication that

![FIGURE 4. Stress/inflammation-regulated MAP3Ks. A: complex regulation of MEKs by members of the MEKK family. Some MEKKs may also regulate NF-κB via phosphorylation of IkB kinase (IKK). B: same as A, except these are distinct MAP3Ks that are not considered members of the MEKK group. C: parallel functions of MEKK1 in ubiquitination. MEKK1, through its RING domain, can apparently directly ubiquitinate ERK1 fostering its degradation. MEKK1 also binds the E3 ligase Itch through which it can foster ubiquitination and degradation of JunB (as well as c-Jun as indicated in FIGURE 2B). This MEKK1/Itch-dependent degradation of c-Jun and JunB is prominent in T lymphocytes and blunts T_{h}2 cell functions. This contrasts with MEKK1/Itch-dependent c-Jun ubiquitination, which is important to T_{h}2 cell tolerogenesis (FIGURE 2C). D: regulation of Tp1-2 by IKK and p105. Tp1-2 is restrained by the NF-κB regulatory subunit p105. IKK phosphorylates and triggers the ubiquitin-dependent degradation of p105. Tp1-2 is liberated and autophosphorylates, becoming active.](http://physrev.physiology.org/
the SWIM domain may be involved also in E3 ligase activity. Thus MEKK1-related protein X (MEX) is an E3 ligase structurally similar to the PHD/RING-SWIM domain region of MEKK1 (MEX does not have a kinase domain). Structure function studies of MEX have revealed that the SWIM domain is essential to MEX's E3 ligase activity, suggesting that this motif in MEKK1 may play a similar role (210).

MEKK1 can activate MKK4 and MKK7, and through these, the JNKs. MKK4 is the preferred MEKK1 substrate, and accordingly, MEKK1 is a relatively selective activator of the JNKs (FIGURE 4). However, in some instances, MEKK1 can also activate the ERKs and p38 (sect. III D1, TABLE 1) (338). MEKK1 can be activated, in cultured cells, by TNF (10), microtubule poisons, oxidant stress (198), and certain receptor Tyr kinase agonists (76). Of particular note, MEKK1 is activated in a complex manner by the B lymphocyte receptor protein CD40. Activation of MEKK1 by CD40 is key to CD40 recruitment of MAPKs (190) (sect. III D1).

B) MEKK-2 AND -3: MORE PROMISCUOUS ACTIVATORS OF THE JNKs, P38S, AND ERKS. MEKK-2 and -3 (MAP3K-2 and -3, respectively) are essentially a small MEKK subgroup sharing a closer sequence similarity within their kinase domains (>90% identity) than with that of MEKK1 (~65% identity). MEKK2 and MEKK3 are also considerably smaller than MEKK1 (~70 and 71 kDa, respectively). In addition, the noncatalytic portions of MEKK2 and -3 are quite similar. The MEKK2 and MEKK3 catalytic domains are COOH terminal (AAs 362–619 for MEKK2, AAs 368–707 for MEKK3). The NH2-terminal noncatalytic portions of MEKK2 and -3 contain no motifs indicative of function or regulation (16, 71) (FIGURE 4A).

Upon overexpression, MEKK-2 and -3 each can activate the ERK pathway (via MEK1) and the JNKs and p38s (through, respectively, MKKs-4/7 and 3/6) (16, 55, 71, 72). Indeed, genetic studies suggest that MEKK3 is a physiological MKK6-kinase (120). Biochemical and some genetic studies have also shown that MEKK3 can, in response to TNF, activate the NF-kB pathway via direct activation of the 1kB kinases (IKKs) (120) (FIGURE 4A, TABLE 1). MEKK-2 and -3 have been implicated in Treg and TH17 cell differentiation (29) (sect. IVC4), and MEKK3 is important in cardiac development (340) (sect. IVB3).

C) MEKK4: ACTIVATION OF JNK AND P38 PATHWAYS. The ~150-kDa MEKK4 (MAP3K4, TABLE 1), like MEKK1, consists of a COOH-terminal kinase domain (AAs 1337–1597) and an extensive NH2-terminal regulatory region with numerous potential protein interaction motifs. Among these are a putative binding site for SH3 domain-containing proteins (AAs 27–38) (95, 283), a region required to bind proteins of the growth arrest and DNA damage (GADD)-45 family (AAs 147–250) (284), and a putative PH domain (AAs 225–398) (95, 283). The MEKK4 kinase domain shares ~55% amino acid homology with that of MEKK-1, -2, and -3. There is some uncertainty as to the precise substrate spectrum for MEKK4, with results suggesting that MEKK4 is selective for MKK4 and the JNKs in vivo and in vitro (95). In contrast, others have shown that MEKK4 can activate MKK4 as well as MKK-3 and -6 (283). Still others have reported that MEKK4 is selective for the p38 pathway in vivo (35). The reason for these discrepancies is unclear. MEKK4 may be an effector for stress pathways activated by genotoxins and has been linked to T(H)1 cell maturation (see sect. IVE3).

3. Other MAP3Ks

A) TAK1 RECRUITS JNK, P38, AND NF-kB IN RESPONSE TO BOTH PROINFLAMMATORY CYTOKINES AND TGF-β FAMILY CYTOKINES. The ~60-kDa TGF-β-activated kinase-1 (TAK1, MAP3K7) consists of an NH2-terminal kinase domain (AAs 30–294) preceded by a short regulatory motif (AAs 1–22) and followed by a COOH-terminal extension (AAs 295–557), the function of which is unclear. The short NH2-terminal motif appears to serve an inhibitory role (336).

Two hybrid screening with TAK1 AAs 1–22 as bait identified TAK1 binding proteins (TAB)-1 and -2 were identified in two hybrid screens that employed TAK1 AAs 1–22 as bait. These two regulatory proteins along with a third TAB protein (TAB3) are essential for coupling TAK1 to upstream signals (see section III D) (34, 127, 134, 207, 266, 281, 304). Endogenous TAK1 is activated by TGF-β, IL-1, TNF, and PAMPs. In vitro and upon overexpression in situ, TAK1 can phosphorylate and activate MKK-3, -4, and -6. TAK1 can also phosphorylate and activate the IKKs, and there is considerable evidence that TAK1 is a physiologically relevant IKK activator (127, 140, 201, 207, 304, 332, 336) (FIGURE 4B).

TAs-2 and -3 confer a unique mode of regulation on TAK1 wherein the enzyme can be activated by free and anchored Lys63-linked polyubiquitin chains synthesized in a stimulus-dependent manner by members of the TNF receptor-associated factor (TRAF) family of E3 ubiquitin ligases (140, 304, 332) (sect. III D, FIGURE 8B). Genetic studies of TAK1 suggest a prominent role for TAK1 in the recruitment of NF-kB by stimuli that recruit TRAF proteins (140, 287, 303, 304, 332). A physiologically relevant role for TAK1 in JNK and p38 activation is likely as well, at least in certain cell types (287, 303) (FIGURE 4B, sect. IVC1).

B) ACTIVATION OF BOTH THE JNKs AND THE P38S BY ASK1 AND ASK2. Apoptosis signal-regulating kinases-1 and -2 (ASK1, also called MAP3K5, MAPKKK5; ASK2 is also called MAP3K6; TABLE 1) are a pair of very closely related ~150-kDa polypeptides. The ASKs contain centrally located kinase domains (ASK1 AAs 677–936) as well as NH2-termi-
nal (ASK1 AAs 1–676) and COOH-terminal (ASK1 AAs 937–1375) extensions, both of which have been implicated in binding polypeptides of the TNF receptor-associated factor (TRAF) family. The NH2-terminal extensions also bind redox sensing enzyme thioredoxin. The ASKs can activate MKK-4, -3, and -6 (125, 179, 211, 245, 309) (FIGURE 4B).

ASK2 is more restricted in its expression than ASK1, with ASK2 expressed primarily in the skin and gut epithelium while ASK1 is ubiquitously expressed. Evidence indicates that ASK-1 and -2 function as a heteromer. Indeed, disruption of ask1 leads to constitutive degradation of ASK2; thus ASK2 cannot function biochemically in the absence of ASK1. ASK2’s requirement for ASK1 does not depend on ASK1’s kinase activity, and indeed, ASK2 can be stabilized by kinase-dead ASK1. In contrast, ASK1 can function alone (126, 282) (see also sect. IVD7).

Endogenous ASK1/2 are activated by oxidant stress as well as by TNF. Indeed, the activation of ASK1/2 by TNF is dependent on TNF-induced generation of reactive oxygen intermediates (ROIs) (100, 125, 179, 211, 245, 292). ASK1/2 are likely to be an important effectors coupling these agonists to the JNKs and p38s.

Despite their close structural similarities and the dependence of ASK2 on ASK1, ASK1 and ASK2 perform specific, nonredundant functions in vivo where they regulate distinct aspects of cell survival and inflammation in tumorigenesis (126) (see sect. IVD7).

C) TPL-2, AN IMPORTANT PROINFLAMMATORY ERK PATHWAY ACTIVATOR AS WELL AS AN ACTIVATOR OF THE JNK AND P38 PATHWAYS. Tumor progression locus-2 (Tpl-2), also called Cot and MAP3K8 (TABLE 1), is a 58-kDa protein Ser/Thr kinase with a central kinase domain (AAs 139–394), similar to S. cerevisiae STE11, a COOH-terminal regulatory domain (AAs 395–467) and an NH2-terminal domain, of unknown function, which is truncated in some mRNA splicing isoforms to yield a 52-kDa form (25, 219). Tpl-2 was originally identified as an oncogene activated by insertional mutagenesis. Thus moloney leukemia virus proviral insertions atTpl-2, which always target the last intron of the gene, elicit the enhanced expression of a COOH terminally truncated, constitutively active protein the expression of which is enhanced, both through elevated 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 (25).

When transiently overexpressed, Tpl-2 activates the JNKs and ERKs with equal potency (25, 249), and Tpl-2 can directly activate MEK1 and MKK4 in vitro and in transfected cells (249). However, ERK activation is likely to be more physiologically relevant (69) (FIGURE 4B).

Activation of Tpl-2 in macrophages by TNF and PAMPs follows an unusual mechanism. In resting cells, the Tpl-2 58- and 52-kDa isoforms exist as a heteromer in complex with the p105 NF-kB1 subunit and a second protein, A20-binding inhibitor of NF-kB2 (ABIN2). Administration of agonist (TNF, LPS) results in phosphorylation of p105 at Ser931, Ser923, and Ser932, a reaction catalyzed by the IkB-kinase-β (IKKβ). A separate poorly understood kinase phosphorylates the 58-kDa form of Tpl2 at Thr290. Phosphorylation of p105 results in ubiquitination and proteosomal degradation of p105, dissociation of ABIN2, and liberation of Tpl2 p52 and p58 heteromers, which auto-phosphorylate at Ser62 becoming active. ABIN2 stabilizes inactive Tpl-2, but Thr290 and Ser62 phosphorylation of Tpl-2, and consequent dissociation of ABIN2, while resulting in Tpl-2 activation, also renders Tpl-2 susceptible to subsequent ubiquitination and proteosomal degradation, thus providing for feedback regulation of this pathway (12, 218, 273) (FIGURE 4C). Genetic disruption studies reveal cell-dependent Tpl-2 functions, notably in TRAF-dependent activation of ERK (69) (sect. IVCI).

D) THE MIXED LINEAGE KINASES ARE POTENT AND SELECTIVE ACTIVATORS OF JNK AND P38 PATHWAYS. The mixed lineage kinases (MLKs) are a small family of protein Ser/Thr kinases with a common structural configuration. An NH2-terminal kinase domain is followed by one to two leucine zippers, a Cdc42/Rac interaction and binding (CRIB) domain, and a COOH-terminal proline-rich domain with several consensus SH3 binding motifs. The MLKs are clearly Ser/Thr specific. Despite this, as their names suggest, the kinase domains of the MLKs contain structural features found in both Ser/Thr and Tyr kinases. Four MLKs have been well described and are clear activators of MAPK pathways: MLK1, MLK2 (also called MKN28 cell-derived Ser/Thr kinase, MST, TABLE 1), 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 1). In addition to the common features described above, MLK2 and MLK3 contain SH3 domains NH2 terminal to their kinase domains (68, 89, 90, 114).

MLK-1, -2, and -3 as well as DLK (MAP3K-9, -10, -11, and -12, respectively) are potent activators of MKK4 and especially MKK7, and through these, the JNKs. MLK3 can also activate the p38s via activation of MKK3 and MKK6 (FIGURE 4B). The ERK pathway is not a strong MLK target, although MEK1 can function as a MLK substrate in vitro (90, 109, 110, 230, 291). MLKs may function in innate immunity and metabolic regulation (see sect. IV, CI and E5).

E) TAO KINASES SHARE STRUCTURAL SIMILARITY TO BOTH STE20 AND MAP3KS YET FUNCTION AS MAP3KS SELECTIVELY UPSTREAM OF THE P38S. The thousand-and-one (TAO) kinases (TAO1 and
TAO2) are a pair of 1,001-amino acid Ser/Thr kinases. Interestingly, the TAO kinase domains are structurally similar 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 (32, 108, 124). Not surprisingly, the TAO kinase domains are also significantly similar to mammalian Ste20s of the germinal center kinase group (43% identity with GCK) (see sect. IIIC) (32, 50, 108, 124, 199). However, there is also appreciable identity with the MLK2 kinase domain (33% overall), especially within the substrate binding motifs (124). Prostate-derived STE20-like kinase (PSK, TABLE 1) is a putative splicing isofrom of TAO2. It contains an extended COOH-terminal tail but is otherwise identical to TAO2 expressed at elevated levels in prostate tumors (199). The TAO kinase domains are NH2 terminal and are followed by very large (700 AA) COOH-terminal extensions, the functions of which are unclear.

Despite their similarities to the Ste20/GCKs, the TAOs are direct, specific activators of MKK3, and through MKK3, the p38s (124) (FIGURE 4B). The ability of TAO1 to catalyze directly the activation of MAP2Ks may be due to the kinase domain homology with MLK2 in the substrate binding region. Of note, the remarkable in situ selectivity of the TAOs for MKK3 may be due to the presence of a specific MKK3 docking site (AAs 315–451 on TAO2) (32). Interestingly, this region is rich in acidic amino acid residues, a property shared with MAPK CD loops which bind to basic MAPK interaction motifs on MAPK regulators and substrates (see sect. IID3) (15, 288, 289).

### III. SIGNALING COMPONENTS THAT RECRUIT STRESS-ACTIVATED MAPK CORE PATHWAYS

Sections I and II touched on the subject of mammalian stress-activated MAP3K-MAP2K-MAPK core signaling modules. Here we describe some of the known proximal signaling components thought to couple these core modules to upstream regulators.

#### A. Rho Family GTPases Can Activate the JNKs and p38s

Members of the Ras superfamily of monomeric GTPases are key proximal activators of signal transduction pathways. Ras itself is central to the activation, by mitogens, of the ERK pathway. The regulation of Ras by receptor tyrosine kinases, and the consequent recruitment by Ras of the Raf family of MAP3Ks have been extensively reviewed (23, 192, 201, 203). The paradigm of Raf regulation points to three crucial events governing MAP3K activation: 1) binding to an upstream activating protein and consequent membrane translocation, 2) phosphorylation, and 3) homoligomerization (236, 317, 350). These steps may be important in the regulation of MAP3Ks recruited by stress and inflammation.

At least some stress-activated MAPK core pathways are putative targets of members of the Rho subfamily of the Ras superfamily. Mammalian Rho family GTPases include the Rho (RhoA-E), Rac (Rac1 and -2), and Cdc42 (Cdc42Hs, G25K, Tc10, and Chp) groups. As with all Ras superfamily members, Rho subfamily GTPases are active in the GTP-bound state and inactive in the GDP-bound state (130). Rac1 and Cdc42 are Ras effectors and may link JNK or p38 to agonists that recruit Ras, including some mitogens (130).

When overexpressed, constitutively active, GTPase-deficient (Val12) mutants of the Rac and Cdc42 groups, but not the Rho group, are vigorous activators of the JNKs and p38s (130). Consistent with these findings, expression of the Dbl family Rho guanine nucleotide exchange factors that recruit Rac and Cdc42, also results in strong JNK activation (130). The so-called Cdc42/Rac interaction and binding (CRIB) domain, which interacts directly with the effector loops of Rac1 and Cdc42Hs, is present in most, but not all, Rac and Cdc42 targets (20). These include the MAP3Ks MEKK4 and MLK-2 and -3. However, the precise effectors that couple Rho GTPases to JNK and p38 remain to be identified unambiguously through genetic studies.

The CRIB motifs of the MAP3Ks MLK2 and MLK3 enable these MAP3Ks to interact directly with Cdc42Hs and Rac1 in a GTP-dependent manner in vitro, a property not demonstrated for MEKK4. However, although MLK2 and -3 may be Cdc42Hs effectors (below), it is unlikely that they are direct Rac targets. In support of this, mutation of the effector loop of Rac1 Phe37 to Ala has no effect on recruitment of the JNK pathway in situ. However, neither MLK3 nor MLK2 can interact with Phe37Ala-Rac1 in vivo (167, 290). As discussed below, Phe37Ala-Rac1 can bind the scaffold protein plenty of SH3 domains (POSH), which may serve to couple Rac1 to the JNKs (290) (FIGURE 5).

In contrast, MLK3 may be a Cdc42Hs effector. The leucine zipper of MLK3 (see sect. IIF3, AAs 400–487) is critical for homodimerization, which, in turn, is central to MLK3’s ability to signal downstream to MKKs (89, 175). Of particular importance, coexpression of MLK3 with Val12-Cdc42Hs significantly increases MLK3 to homodimerization (175), suggesting that GTP-Cdc42Hs binds and promotes MLK3 (or MLK2) homodimerization and activation (FIGURE 5).

#### B. Several Scaffold Proteins Regulate the JNKs

Insofar as mammalian cells express multiple different MAP3Ks and additional upstream regulators of the JNKs

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and p38s, to maintain signaling specificity, efficiency, and integrity, it is imperative that individual MAP3K-MAP2K-MAPK core signaling components be organized into groups that are both subject to regulation by select activators and able to recruit select effectors. This sequestration is mediated by scaffold proteins. These proteins collect specific MAP3K-MAP2K-MAPK core modules into organized signaling oligomers.

1. JIPs: a family of scaffold proteins that may regulate JNKs by nucleating core MLK-MKK7-JNK signaling complexes

JNK interacting protein-1 (JIP1), a ~60-kDa polypeptide, was the first discovered member of a family of mammalian scaffold proteins that regulate JNK signaling. The JIP1 NH$_2$-terminal domain (AAs 143–163) selectively binds the JNKs. The JIP1 COOH-terminal extension includes an SH3 domain (AAs 491–600). Between these is a proline-rich segment (AAs 281–448) that contains several motifs that conform to the consensus for SH3 binding sites (63).

JIP1 interacts with kinases at all three levels of JNK core signaling modules. Thus JIP1 binds the MAP3Ks MLK3 and DLK (but not MEKK1), the MAP2K, MKK7 (but not MKK4), and JNK1. It remains to be established whether this binding is at all sequential or regulated in any way. The JIP1 SH3 domain is required for MLK3 binding, while a segment of the JIP central region (AAs 283–471) that overlaps with the proline-rich region is needed for MKK7 binding. JIP1 also interacts with hematopoietic progenitor kinase-1 (HPK1), a mammalian germinal center kinase (GCK) homolog, but not GCK-related, another GCK homolog (see sect. IIIC) (63, 323). The binding of HPK1 is probably indirect, via the binding of HPK1 to MLK3. The ability of JIP1 to potentially link HPK1 to a complete JNK-activating core signaling module corresponds well with the idea of JIP1 as a true scaffold protein. JIP1 is heavily expressed in the brain. Disruption of jip1 indicates a role in stress responses, notably apoptosis in response to excitotoxic stimuli (324). JIP1 has also been linked to cortical neuron axonal development (48); JNKs also have been linked to the promotion of neuronal development (sect. IV A).

JIP2 is a second JIP family member. It is quite similar structurally and functionally to JIP1, and, like JIP1 is almost exclusively localized in brain. Accordingly, JIP2 can bind in situ with MLK-2 and -3 (but not MEK family MAP3Ks), MKK7 (but not MEK1, MKK3, MKK4, or MKK6) and JNKs 1–3 (but not p38 or ERK). Both JIP1 and JIP2 preferentially interact with JNK1 over JNK-2 or -3, and both JIP1 and -2 can in overexpression studies potentiate JNK activation by coexpressed MLK3. In addition, JIP1 and JIP2 (endogenous and ectopically expressed) can homo- and het- erosdimerize. However, JIP1 binds all JNK isoforms more strongly than does JIP2 (345).

JIP3 is a large polypeptide (~144 kDa) expressed selectively in brain and heart. JIP3 consists of a leucine zipper motif (AAs 392–427) as well as several coiled-coiled motifs. JIP3 can bind all three JNK isoforms, but, as with JIP1 and JIP2, binding to JNK1 is strongest. JIP3 binds neither ERKs nor p38s. The JNK binding region spans AAs 202–213, a region conserved with a segment of the JIP1 JNK binding domain (AAs 153–164). This conservation suggests a consensus JNK binding sequence (Arg-X-X-Arg-Pro-Thr-Ser/Thr-Leu-Asn-Va/Leu-Phe-Pro, where X is any amino acid) (128, 148) notably similar to the basic MAPK docking sites, present in many MAPK substrates, that bind to MAPK CD motifs (section II D3, TABLES 2 and 3) (15, 288, 289). Recent genetic studies indicate a critical role for JIP3 in brain development, again a function also linked to the JNKs (149) (sect. IV A).

JIP4 is a fourth member of the JIP family. It is structurally similar to JIP3. Interestingly, however, JIP4 appears not to regulate JNK. Instead, biochemical studies indicate that JIP4 regulates the p38s in a mechanism that requires MKK3 and MKK6 (150).

2. POSHs

Plenty of SH3 domains (POSH) 1–3 (also called SH3 do- main containing ring finger, SH3RF 1–3) comprise a family of ~90-kDa polypeptides. POSH proteins each contain four SH3 domains (AAs 139–427, 198–254, 457–511, and 838–892 for POSH1), several clustered polyproline SH3 binding sites (AAs 368–405 for POSH1), and a RING do-
main with E3 ubiquitin ligase activity (AAs 12–52 for POSH1). Interestingly, POSH proteins contain only partial CRIB motifs (localized to AAs 292–362 in POSH1), but can still interact directly with Rac1 (but not Cdc42Hs, Ras, or Rho). Ectopically overexpressed POSH1 stimulates robust JNK and NF-κB activation as well as apoptosis (144, 290, 326).

Effector loop mutants of Rac1 recruit POSH1, a process that correlates tightly with the relative ability of these Rac1 mutants to activate the JNKs (FIGURE 5). Thus POSH1, unlike MLK-2 and -3, can interact with Phe37Ala-Rac1, a Rac1 effector mutant that does not provoke lamellipodium formation but does activate coexpressed JNK (167, 290). In contrast, POSH1 cannot interact with a second Rac1 effector loop mutant, Tyr40Cys Rac1, which does not activate the JNKs in situ (168, 303). Thus, although the MLKs, which contain complete CRIB motifs, the POSHs, but not the MLKs, are likely direct effectors for Rac1 activation of the JNKs, the MLks may be effectors for Cdc42Hs. Cdc42Hs does not interact with POSH proteins. Still, the SH3 domains of POSH may function as scaffold proteins, binding MLKs, that contain SH3 binding sites.

The function of the POSH E3 ligase activity is obscure. POSHs can autoubiquitinate, a function which may serve to suppress POSH-dependent apoptosis (144). In this capacity, POSHs can trigger JNK activation and JNK-dependent apoptosis, especially in neuronal cells where POSH1 proteins may function in the pathogenesis of Parkinson’s disease (144, 326).

3. Actin binding protein-280 (ABP-280), a 280-kDa putative scaffold protein that may regulate TNF and lysophosphatidic acid activation of melanoma cell JNKs

The 280-kDa actin binding protein ABP-280 (also called filamin) crosslinks actin filaments into orthogonal arrays, thereby contributing to the structure of the cortical actin meshwork (277). ABP-280 may also be a scaffold protein for cytokine regulation of JNK.

In situ and in vitro, ABP-280, through its COOH-terminal region (AAs 2282–2454), interacts with MKK4. MEK1, ERK1, and p38a do not interact with ABP-280. MKK4, in turn, indirectly recruits MEKK1 and JNK to ABP-280, and these polypeptides can be isolated with ABP-280 only if MKK4 is present (188). MKK4 can itself act as a scaffold protein (see below) (330). Thus the formation of MEKK1-MKK4-JNK-ABP-280 complexes may enable the recruitment of JNK by selective upstream stimuli. ABP-280 loss of function mutations indicate that ABP-280 is important for the activation of MKK4 and the JNKs in intact melanoma cells, in response to certain specific extracellular stimuli, notably TNF (188).

The proinflammatory adapter protein (sect. IIID) TNF receptor-associated factor-2 (TRAF2) can also interact with ABP-280 through a process that requires the RING and Zn finger domains of TRAF2. As with TNF activation of JNK, activation of JNK by ectopically expressed TRAF2 is also attenuated in melanoma cells bearing ABP-280 loss of function mutants (173). This finding fits with the idea that ABP-280 assembles a TNF responsive signaling complex in these melanoma cells.

4. The intrinsic scaffold properties of MKK4

MKK4, in addition to phosphorylating and activating JNK and p38, functions as a scaffold protein. The scaffolding properties of MKK4 enable it to form selective, dynamic complexes with both an upstream activator (MEKK1) and a substrate (JNK). Thus MEKK1 interacts with the NH2-terminus (AAs 1–77) of inactive, dephosphorylated MKK4. Of note, because MEKK1 does not readily interact in vivo or in vitro with MAP2K-1 or -2, MKK-3 or -6 or with MKK7, none of these MAP2Ks is as good an in vitro substrate of MEKK1 as is MKK4 (330).

Upon the addition of ATP to the MKK4-MEKK1 complex, in vitro, MEKK1 catalyzes the phosphorylation and activation of MKK4, which, in turn, fosters the dissociation of the MKK4-MEKK1 complex (343). The phosphorylated, active form of MKK4 (but not inactive MKK4) then enters into a second specific complex, with JNK, again mediated by the MKK4 NH3-terminal MEKK1 binding domain. Importantly, the MEKK interaction motif also contains a polybasic MAPK docking site that interacts specifically with the JNK CD domain (15, 288, 289). This second complex dissociates upon phosphorylation and activation of JNK (330).

C. The Germinal Center Kinases/Map4ks as Regulators of JNK and p38 Core Signaling Pathways

1. General considerations

The germinal center kinases (GCKs) are a family of Ser/Thr protein kinases that possess NH2-terminal kinase domains distantly related to that of S. cerevisiae Ste20p (TABLE 1). Of note, however, the kinase domain of Ste20p (as well as those of the more closely related mammalian PAKs) is COOH terminal. Some members of the GCK family are selective activators of the JNKs and p38s (50, 58). The founding mammalian member of the GCK family is GCK itself.

The kinase domains of the GCKs are followed by extensive COOH-terminal regulatory domains (CTDs) (50). The GCKs are effectively assembled according to sequence similarity of the catalytic domains into seven subfamilies that...
within each also show marked similarities in their COOH-terminal noncatalytic segments; moreover, each of these subfamilies has a single homolog in *Drosophila* or *Caenorhabditis elegans* (50, 58). The mammalian members of the group I GCK subfamily, i.e., GCK, GCKR(KHS), GLK, and HPK1, have each been shown to act as upstream activators of JNK in some context. In addition, the group IV GCK subfamily, NIK, TNIK, and NRK, also function as upstream activators of JNK (TABLE 1) (50, 58, 64, 82, 119, 139, 146, 154, 261, 279, 296, 344). Numerous studies indicate that those GCKs that recruit the JNKs/p38s do so by recruiting MAP3Ks (27, 64, 82, 119, 139, 146, 154, 261, 279, 296, 344). Accordingly, members of the GCK family have been designated MAPK-kinase-kinase-kinases (Map4ks) by Human Genome Project (TABLE 1), in spite of the fact that little clear evidence exists showing that GCKs can activate MAP3Ks by phosphorylation (27).

The CTDs of those GCKs that recruit the JNKs and p38s are broadly similar, typically including at least two proline/glutamic acid/serine/threonine (PEST) motifs and two or more polyproline putative SH3 domain binding sites. The CTDs of JNK/p38-activating GCKs also include a conserved citron homology domain (CNH) that resembles a similar region in Citron, a conserved metazoan kinase involved in the control of the actin cytoskeleton (50, 58, 64, 82, 119, 139, 146, 154, 261, 279, 296, 344).

GCKR, GLK, NIK and, possibly, TNIK are all activated in situ by TNF (64, 82, 119, 139, 146, 154, 261, 279, 296, 344). Inasmuch as GCKs 1 potently activate the JNKs/p38s and 2) stably associate with core signaling module elements and their upstream activators, it is reasonable to propose that the GCKs coordinately regulate MAPK core signaling modules in conjunction with additional upstream elements.

2. The group I (GCK, GCKR, GLK, and HPK1) and IV (NIK, TNIK, NRK, and MINK1) subfamilies of GCKs/Map4ks each can activate JNK and p38

A) GCK. Transient overexpression of GCK (Map4k2, TABLE 1) results in robust activation of JNK and p38. Two immediate downstream targets of GCK are MLK2 and -3. In vitro, GCK can activate MLK3 and MEKK1 (27).

B) GCKR. The primary sequence of GCKR (Map4k5, also called kinase homologous to STE20, KHS, TABLE 1) is ~60% identical to that of GCK, especially within the CNH motif of the CTD (50, 58, 261, 296). GCKR is a highly selective activator of the JNKs and, when ectopically expressed does not activate the ERKs, p38s or NF-κB (261, 296).

TNF, CD40, and ultraviolet radiation all can activate endogenous GCKR (261, 262), and there is evidence that GCKR is an important effector for TNF, at least in some cell types. Thus GCKR antisense constructs, when expressed in 293 cells, strongly block TNF activation of the JNKs (261).

Interestingly, RNAi studies have also revealed a role for GCKR in both canonical and noncanonical Wnt signaling, at least in B lymphocytes (263). Wnt agonists bind to a class of seven transmembrane receptors in the Frizzled family and play an important role in cell fate decisions and oncogenesis (see also section IVB1). Canonical Wnt signaling involves receptor mediated stabilization of β-catenin. This stabilization requires signal-dependent inhibition of GSK3-catalyzed phosphorylation of β-catenin and enables β-catenin to accumulate and migrate to the nucleus where it dimerizes with TCF transcription factors to regulate gene expression (99).

Noncanonical Wnt signaling is mediated by the Frizzled-linked adapter protein Dishevelled and triggers JNK activation. Mechanisms controlling this process are poorly understood. Disruption or silencing of gckr substantially impairs canonical and noncanonical Wnt3a activation of JNK, GSK3-β phosphorylation/inhibition, and consequent stabilization of β-catenin (263). This result suggests that GCK has functions outside of JNK/MAPK regulation.

C) GLK. As with GCK and GCKR, GLK (Map4k3, TABLE 1) is a potent and selective activator of the JNKs. Moreover, the GLK CTD, especially within the CNH motif, is strikingly similar to those of GCK and GCKR. Endogenous GLK is apparently activated in situ by TNF (64); however, the physiological functions of endogenous GLK are unknown.

D) HPK1. While GCK, GCKR, and GLK share close structural similarity, HPK1 (Map4k1, TABLE 1), a fourth group I GCK, while sharing the broad structural features of JNK and p38-activating GCKs, is structurally more distant from GCK than is GCKR and GLK. The CTD of HPK1 includes four consensus SH3 domain binding sites, the COOH terminal, most of which interacts with the SH3 domain of MLK3 (sect. IIF3). Ectopically expressed HPK1 vigorously activates the JNKs while activating p38 much more weakly. No ERK activation is observed upon HPK1 overexpression (50, 58, 119, 154).

The second and fourth SH3 binding motifs of HPK1 enable HPK1 to bind to the closely related SH2/SH3 adapter proteins Crk and CrkL, an interaction that enables the EGF-stimulated recruitment of HPK1 to the EGF receptor, at least in transfected cells. The Crk(CrkL)-HPK1 interaction may be physiologically relevant insofar as coexpression of Crk or CrkL with HPK1 activates the kinase activity of HPK1 and, in situ triggers synergistic JNK activation (176). Although ectopically expressed HPK1 can interact with MLK3 in situ, physiological effectors linking HPK1 to MAPKs have not been identified genetically or through
RNAi (154). HPK1 may also have several non-MAPK-related functions relevant to the negative regulation of TCR signaling (sect. IV C1).

**E. NIK** (Map4k4, also called HPK1/GCK-like kinase, HGK, **TABLE 1**) and its close relatives TNIK (below) and Nrk cluster into the group IV GCK subfamily, a subfamily distinct from the group I GCKs and most closely related to the *Drosophila* kinase *Misshapen*. However, as with the group I GCK subfamily, transient overexpression of NIK strongly activates the JNKs (279, 344).

NIK contains two consensus SH3 binding motifs (AAs 574–579 and AAs 611–616), both of which can interact with the SH3 domains of the SH2/SH3 adapter protein Nck (279). The significance of the interaction between NIK and Nck is unclear. Overexpression studies suggest a link between NIK/Nck and the Eph Tyr kinases in the recruitment of JNK (11). In addition, given that Nck is thought to be involved in the regulation of the actin cytoskeleton, one function of NIK may be to regulate cell motility and shape. Indeed, *Drosophila Misshapen* is required for embryonic dorsal closure (280). NIK can be activated by TNF (344). *Misshapen* is an effector for *Drosophila* TNF receptor-associated factors (TRAFs; sect. IIID), suggesting a similar role for NIK (178, 262, 346).

**F. TNIK.** TRAF2 and Nck-interacting kinase (TNIK) is ~90% identical, at the amino acid level within the kinase domain (AAs 1–306), ~90% identical within the Leu-rich and CNH motifs (AAs 1019–1360), and ~53% within the remainder of the CTD to NIK. Predictably, therefore, TNIK overexpression results in robust and selective JNK activation. As also expected, TNIK can interact with Nck in situ, although the functional significance of this interaction is unclear. TNIK also interacts with coexpressed TRAF2. That said, there is no evidence that TNIK is recruited by stimuli that signal through TRAF2 (82).

### D. Adapter Proteins That Couple to TNF Receptor Family Receptors and Receptors of the IL-1R and TLR Group Are Crucial Regulators of the JNKs and p38s

Upon binding agonists, transmembrane receptors of the tumor necrosis factor family (the TNFR family) and the IL-1 receptor/TLR family, as well as cytosolic receptors of the nuclear binding/oligomerization domain-leucine-rich repeat (NOD-LRR) and retinoic acid-inducible gene (RIG)-like-helicase (RLH) families provoke a suite of diverse inflammatory responses. As such, these receptors are crucial to immune cell development, innate and acquired immunity, and the pathogenesis of a number of diseases such as cancer and type 2 diabetes mellitus (see section IV). Thus these receptor families are among the most important activators of the JNKs, p38s. Although widely divergent structurally, the basic mode of signaling for these receptor families is quite similar; these receptors do not possess intrinsic enzymatic activity relevant to signaling. Instead, the receptors signal by recruiting intracellular adapter proteins (19, 40, 147, 151, 195). The repertoire of adapter proteins used by these receptors, as well as the spatial and temporal association of these adapters and the downstream effectors they target are complex, cell specific, and still incompletely understood.

The TRAFs are critical proinflammatory signal transducing adapter proteins. As such, most proinflammatory receptor signaling pathways converge on one or more of the seven known TRAF proteins which, in turn, recruit key downstream signaling components. Genetic studies indicate that TNF signaling to MAPKs requires TRAF2, while IL-1 and PRR signaling to MAPKs requires TRAF6. TRAF2 also relays endoplasmic reticulum stress signals to the JNKs (7, 103, 222).

The TRAFs consist of two closely spaced 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. The TRAF domains are responsible for binding to a subset of TRAF effectors and for binding upstream activator adapter proteins such as TNF receptor associated death domain (TRADD) for the TNF pathway, and myeloid differentiation factor-88 (MyD88) for the TLRs. The function of the Zn finger domains is unclear; however, the RING fingers possess E3 ubiquitin ligase activity and are important for the activation of downstream effectors (7, 103, 213, 222).

A recent series of elegant studies has identified the a major mechanism by which TRAF proteins signal. The RING domains of TRAFs enable autoubiquitination, generating poly-Ub chains linked at Ub-Lys63. Of particular importance, TRAFs can also generate free Lys63-linked poly-Ub chains which act as second messengers recruiting the TAB2-TAK1 complex. This complex is required for efficient activation by IL-1 and PAMPs of NF-κB and, at least in some circumstances, JNK and p38 (140, 222, 304, 332) (**FIGURE 6B**).

1. In B cells, the TNF receptor family member CD40 recruits TRAF2 and TRAF3 into a large complex that activates MEKK1. In response to toll-like receptor agonists, MEKK1 may also be activated by partial proteolytic cleavage coupled to the adapter protein ECSIT

CD40 is a receptor present principally on B cells, where it acts to trigger B cell maturation and activation upon engagement by CD40 ligand (CD40L), a membrane-associated agonist present on activated T cells. CD40 is structurally similar to TNF receptors and recruits TRAF proteins for signal transduction. Matsuzawa and colleagues (190)
showed that CD40 engagement triggers the formation of a large complex containing TRAF2, TRAF3, the ubiquitin (Ub) conjugating enzyme Ubc13, the E3 Ub ligases cellular inhibitor of apoptosis proteins clAP1 and clAP2, the 1κB-kinase (IKK) regulatory scaffolding subunit IKKγ, and MEKK1. TRAF2, Ubc13, and IKKγ are required for complexing with MEKK1 and subsequent CD40 activation of ERK, JNK, and p38; however, MEKK1 activation does not occur without CD40 stimulus-dependent dissociation of the complex from CD40 itself, a process that requires prior clAP1/2-dependent degradation of TRAF3 (FIGURE 6C). This complex mode of signaling may enable spatial and temporal regulation of signal transduction. Indeed, CD40 recruits other TRAFs, notably TRAF6, for activation of NF-κB (88, 190).

Evolutionarily conserved signaling intermediate in Toll pathways (ECSIT) is an adapter protein that interacts with the TRAF domain of TRAF6 (but not TRAF2 or -5), and with full-length MEKK1. This interaction apparently stimulates the LPS/TRL4-stimulated partial proteolytic cleavage of MEKK1 into an 80-kDa constitutively active fragment, thereby triggering JNK activation (158). The cell/tissue types in which this reaction occurs are unclear, however. Moreover, recent studies suggest several other pathways coupling TLRs to JNK (sects. IIIIC1 and IVC1). More recent experiments have implicated ECSIT in the LPS-stimulated production of reactive oxygen intermediates (319).

2. GCKs may function with TRAFs to activate MAP3Ks

As discussed in section IIIIC2, GCK, an effector for TRAF6, and an activator of MLK2 and -3, is recruited by PRRs. In contrast, GCKR, GLK, NIK, and TNIK are putative TNF effectors (64, 261, 344). Accumulating evidence indicates also that PRRs and TNFRs link to GCK, GCKR and, possibly, NIK and TNIK through the TRAFs (sect. IIIIC) (82, 262, 350, 351). Thus TNF activates GCKR in a TRAF2-dependent manner and antisense GCKR constructs blunt TNF activation of 293 cell JNK (261, 262).

GCKR can bind TRAF2 in situ while GCK interacts with TRAF6. These interactions require the CNH domains of both kinases (82, 262, 346). GCKR is activated upon coexpression with TRAF2, a process that requires the TRAF2 RING domain (261). In line with this, TRAF2 can also foster the Lys63-linked ubiquitination of GCKR. This is in sharp contrast to the mode of activation of GCK by TRAF6 which occurs independently of the RING domain (sect. IIIIC2).

3. ASK1 is an effector for TRAFs-specifically, a ASK1 is a TNF effector recruited by TRAF2: the role of cellular redox in ASK1 regulation

ASK1 and the closely related ASK2 are MAP3Ks that function upstream of the JNKs (through activation of MKK4) and the p38s (through MKK3 and MKK6). TNF activates the MAP3K activity of ASK1 (125, 126, 282) (sect. IIIF3).

Upon ectopic expression, ASK1 interacts in situ with recombinant TRAF2, -5, and -6, and TNF stimulates a transient interaction between endogenous TRAF2 and ASK1 in situ. Recombinant TRAF2 can also activate coexpressed ASK1 (179, 211).

The regulation of ASK1 by TRAF2 is redox dependent. Thus the TNF-dependent association of endogenous ASK1 and TRAF2 is efficiently attenuated by free radical scavengers (179). ASK1 is also inhibited endogenously by the redox sensing oxidoreductase thioredoxin (Trx). However, for this inhibition to occur, Trx must be in a reduced state. Thus, upon treatment of cells with oxidant stresses (H2O2), and consequent Trx oxidation, ASK1 dissociates from Trx and binds TRAF2. The exchange of Trx for TRAF2 triggers activation of ASK1 (245) (FIGURE 6D). TNF can elicit the production of ROIs, and JNK activation by coexpressed TRAF2 is partially reversed by free radical scavengers (211). Inasmuch as expression of TRAF2, but not a mutant TRAF2 construct missing the RING effector domain, triggers the production of ROI (179), the E3 ligase activity of TRAF2 may contribute to ROI production (245).

4. TAK1 is a target for TGF-β through its association with TAB1, and for PRRs, TNF and IL-1 through its association with TAB2

TAK1 binding protein-1 (TAB1), TAB2, and TAB3 are regulatory subunits that bind and foster activation of TAK1 and its downstream effectors, the JNKs and p38s (34, 127, 134, 140, 266, 281). TAB1 and TAB2 are structurally quite

FIGURE 6. Regulation of proximal components that recruit MAP3Ks. A: regulation of TGF-β-activated kinase-1 (TAK1) by TRAF6-dependent formation of Lys63-linked free polyubiquitin chains. Engagement of the IL-1 receptor recruits TRAF6, activating its autoubiquitination and free Lys63-linked polyubiquitination chain synthesis. Free K63-linked poly-Ub activates TAK1 by binding the regulatory subunit TAK1 associated and binding protein-2 (TAB2). B: CD40L/CD40 recruitment of MEKK1 in B cells. In resting cells, MEKK1 is part of a large complex that includes TRAF3, IKKγ, the E3 ligases clAP1 and -2, and the E2 Ub transfer protein Ubc13. Engagement of CD40 triggers Ubc13/clAP1/2-dependent ubiquitination of TRAF3 and consequent TRAF3 degradation. The remaining complex dissociates from CD40, and the new active MEKK1 phosphorylates substrates. C: regulation of ASK1 by reactive oxygen intermediates (ROIs). Resting cell ASK1 is associated with reduced thioredoxin (Trx). Cytokine-induced ROI production oxidizes Trx leading to its dissociation from ASK1 and to the binding of TRAF2. This activates ASK1. D: T cell receptor engagement activates MEKK4 via promoting an association with GADD45β or GADD45γ. This is key to subsequent activation of p38 and to the propagation of T helper 1 cell functions.
dissimilar. TAB1 is a pseudophosphatase with an NH$_2$-terminal domain of unknown function (AAs 26–323) that is strikingly similar to the catalytic subunit of protein phosphatase 2C. TAB2 and TAB3 contain NH$_2$-terminal coupling of ubiquitin to ER degradation (CUE) domains (AAs 13–55 of TAB2) and COOH-terminal Npl4-Zn finger motifs (AAs 671–695 of TAB2).

There is evidence that, via TAB1, TAK1 couples the JNKs and p38s to TGF-β superfamily receptors (201, 236). In certain cell types, TGF-β and BMPs-2 and -4 can activate the JNKs and p38s; and the MAP3K catalytic activity of murine osteoblastic cell (MC3T3-E1) TAK1 is activated by TGF-β and BMP4. Trimeric complexes of BMPR-I, the E3 Ub ligase XIAP and TAB1 can be isolated (337). XIAP also interacts directly with the type-I receptors for BMPs-2 and -4. Given that XIAP expression in *Xenopus* embryos reproduces the ventralizing effects of BMP, whereas introduction of the TAB1 binding region of XIAP prevents the expression of ventral mesoderm markers elicited by a constitutively active mutant type-I BMP receptor, it is likely that the XIAP-TAB1-TAK1 complex is physiologically relevant (337). This complex may couple TAK1 to TGF-β family receptors.

TAK1 is also strongly recruited by proinflammatory cytokines and PAMPs. This process requires TRAF-catalyzed formation of free Lys63-linked poly-Ub chains (140, 332). Although CUE domains interact with poly-Ub, evidence suggests that it is the Npl4-Zn finger motif of TAB2 that binds poly-Ub. This binding engages the TAB2-TAK1 complex, thereby fostering TAK1 activation (162, 254). The precise mechanism of TAK1 activation remains somewhat obscure, but may involve induced proximity/aggregation (140, 332).

### E. Regulation of MEKK4 by DNA Damage May Involve a Direct Interaction With GADD45 Homologs

The majority of chemotherapeutic and radiation treatment protocols that are used to treat cancer kill cells by producing irreversible DNA damage and consequent tumor cell apoptosis. Unwanted side effects of these treatments include the collateral killing of normal cells both through DNA damage and nonspecific redox stress. To enable the improvement of conventional anticancer treatments, it is important to understand how chemical and radiant genotoxins trigger cell death.

At the cellular level, genotoxic stresses trigger rapid cell cycle arrest, and, to maintain the integrity of the genome, either DNA and general cellular repair, or, if the damage is too great, apoptosis. In certain instances, JNK and p38 have been shown to be activated by genotoxins such as UV and γ radiation, methylmethane sulfonate (MMS), cytosine arabinoside (AraC), N-acetoxy-2-acetylaminofluorene, cisplatinum (CDDP), mitomycin-C, etc. However, the spectrum of genotoxic stresses capable of activating the JNKs, as well as the upstream components that couple these stresses to the JNKs, remain nebulous, a consequence, perhaps, of the nonspecific effects these toxins have on cells (31, 153, 180, 216, 258).

Saito and co-workers (284) showed that MEKK4 is activated by genotoxins that transcriptionally induce proteins of the growth arrest and DNA damage-45 (GADD45) family. GADD genes are rapidly induced by DNA damage and are thought to play a role in coordinating the cellular response to genotoxins. *gadd45* was one of the first GADD genes to be identified (79, 145).

All three *gadd45* homologs (GADD45α, -β, and -γ) can, when ectopically overexpressed, interact with MEKK4 and activate JNK and p38 in a manner reversed by dominant inhibitory MEKK4, and purified GADD45 can activate MEKK4 in vitro (284).

However, it must be noted that it is unclear whether or not, in response to genotoxic stress, GADD45 proteins are induced to sufficient levels to activate MEKK4 at the times when MEKK4 and JNK are activated. Thus neither endogenous GADD45α, -β, nor -γ is present at detectable levels when JNK is fully activated by the DNA damaging chemicals MMS (259, 308). Moreover, JNK is activated by MMS and other genotoxins in *gadd45α−/−* cells. Lastly, ectopic expression of either GADD45α, -β, or -γ does not activate coexpressed JNK (308). Lastly, there is little or no detectable JNK activation in fibroblasts (p53 positive) treated with ionizing radiation sufficient to induce GADD45 expression (259).

Some clarification comes from genetic studies indicating that the GADD45-MEKK4 interaction is relevant, but for p38 activation selectively; and that GADD45-β and -γ are key to MEKK4 regulation. GADD45-α and MEKK4 are also implicated in TCR-mediated p38 activation and T-cell differentiation (35, 250, 251) (FIGURE 6E and sect. IV.C3).

### IV. BIOLOGICAL FUNCTIONS OF THE JNKS AND P38 MAPKS

The pathway architectures and kinase/substrate relationships described above were in many cases first identified using in vitro assays or by transient expression of cDNAs in transformed cells in long-term culture. The results of such experiments identify relationships that are possible, and other kinds of studies are required to ascertain whether such relationships actually occur between the elements when expressed endogenously in an intact cell. A well-characterized cohort of chemical kinase inhibitors is available, and the advent of RNAi-induced mRNA depletion has greatly facil-
1. The JNKs and brain development

Studies of single and combinatorial jnk isoform knockout mice have revealed specific stress response and developmental roles for the different JNK isoforms expressed in the brain.

The glutamate receptor agonist kainic acid rapidly induces seizures resembling those of epilepsy. Kainate also causes the widespread apoptosis hippocampal neurons, a process similar to that which occurs in stroke and Alzheimer’s disease. In contrast to the ubiquitously expressed JNK1 and JNK2, JNK3 is expressed selectively in brain, heart, and testis. Disruption of jnk3 produces animals that are strikingly resistant to kainate seizures (339) and, more importantly, from kainate-induced hippocampal neuronal apoptosis (339). Hippocampal cell death is a major clinical indicator in stroke and Alzheimer’s disease.

The establishment of a role for JNK3 in the brain’s response to stress prompted the investigation of the role of the JNKs in normal brain development and function. Analysis of jnk1/jnk2, jnk2/jnk3, jnk1/jnk3 double-knockout mice revealed that JNK-1 and -2, acting together, play a pivotal role in regional-specific apoptosis during early brain development (161, 240). In contrast, jnk1/jnk3 and jnk2/jnk3 mice are viable. Thus deletion of jnk1 or jnk2 alone is insufficient to affect brain development; moreover, JNK3 is unlikely to be a rate-limiting component in brain development (339).

The combined disruption of jnk1 and jnk2 is embryonically lethal, resulting in an abnormally prominent hindbrain exencephaly. Normally, during the process of cephalic neurulation, the closure of the hindbrain neural tube is a key step, and regulated cell death is important to hindbrain neural tube closure. However, in an indication of reduced apoptotic cell degeneration, jnk1/jnk2 double-knockouts exhibited reduced pyknotic nuclei at the lateral edges of the converging hindbrain, compared with wild-type or single knockout controls (161, 240). Thus appropriate developmental hindbrain apoptosis requires the collaborative function of JNK1 and JNK2.

In contrast to the regulated apoptosis promoted by JNK1/2 in the hindbrain, the jnk1/jnk2 double knockout increased forebrain apoptosis [assayed as pyknotic nuclei or terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assays which serve as an indicator of the internucleosomal cleavage characteristic of apoptotic cells], suggesting instead that the JNKs reduce apoptosis in the forebrain (161, 240). The increased forebrain apoptosis in the jnk1/jnk2 double knockout mice arises from an excessive activation of caspase-3, as detected by increased immunoreactivity for the cleaved, 17-kDa active form of caspase-3, but not its inactive 35-kDa precursor (161, 240).

A. JNKs and the Central Nervous System

Overexpression of JNK in primary neurons or PC12 cells in culture will induce neurite extension, at least in part through phosphorylation of microtubule-associated proteins (28), and activated JNK is required for axonogenesis (212). JNK is activated after injury to central or peripheral neurons. In the periphery, JNK inhibitors suppress cell death; however, they also inhibit the ability of the surviving neurons to regenerate their axons (206). The molecular details (i.e., JNK isoforms, signaling complexes, etc.) that underlie this duality of action remain to be uncovered. Nevertheless, a role for JNK inhibitors in stroke and a variety of inflammatory and degenerative CNS pathologies continues to be actively investigated.

itiated such validation studies, when appropriate measures are used to account for off-target effects. RNAi and chemical inhibitors are most conveniently applied to transformed cell lines in permanent culture. Thus it must be recognized that, in addition to whatever mutations were engendered in vivo in the progression of those cells to the transformed state, the wiring of their signaling pathways may have been altered further in a manner that enables them to survive and proliferate in the nonphysiological in vitro milieu. This caveat does not necessarily invalidate the physiological relevance of epistatic relationships identified in such cells, but strictly speaking limits them to the cell type in which they are demonstrated. Some additional demonstration is needed to establish whether or not such a signaling event occurs only in the context of the transformed state. A relationship so identified may occur universally in all cell types in vivo, or more realistically, may occur only in a tissuespecific and/or developmentally restricted context. Replication of the effect using chemical inhibitors, RNAi-induced mRNA depletion or antisense RNA in primary cells or in living organisms, when technically feasible, provides very strong support. More commonly, gene modification in a model organism is taken as a gold standard. This approach can however be confounded by several mechanisms, e.g., the presence of genes of overlapping or interacting function, whose expression may be altered during development so as to compensate for deletion of the target gene. Such a problem can be mitigated by conditional inactivation of the target gene postdevelopment; this is perhaps the closest mimic of the effects of a druglike modifier of target action. Global gene knockouts present a phenotype that is the sum of the action of that gene product is all tissues, which may not uncover the direct effects of the target in any one tissue. Target actions in one cell type can have indirect effects on a second tissue that differ from the action of the target itself in that second tissue. The gene function in a specific cell type is best revealed by conditional inactivation of the target in a cell type-specific manner. In the examples that follow, the application of all these approaches will be illustrated and their limitations occasionally revealed.
Thus JNK1 and JNK2 perform opposing functions with regard to fore- and hindbrain developmental apoptosis.

In further support for a role for JNKs in brain development, disruption of the gene encoding the neuron-specific JNK scaffold JIP1 attenuates neuronal axon development (48) and neuronal stress responses (324). Disruption of JIP3 strongly impairs development of the telencephalic commissure, a major connection between the left and right brain hemispheres (149). Whether JIP3 also plays a role in the developmental functions of JNKs revealed by the jnk1/jnk2 deletions is unclear.

2. JNKs and the suppression of autophagy in the brain

A more recent study has examined the consequences of neuron-specific JNK1–3 triple deletion. Because the combined disruption of jnk1 and jnk2 is embryonic lethal, specifically causing substantially impaired brain development, Davis and colleagues (334) crossed jnk2−/−;jnk3−/− mice, which are viable, with mice bearing a floxed allele of jnk1 to generate jnk1LoxP/LoxP; jnk2−/−;jnk3−/− mice. Cerebellar granule neurons were isolated from these mice and treated with adenoviral Cre recombinase to disrupt the floxed jnk1 allele, thereby creating triple knockout JNKTKO neurons. Remarkably, the JNKTKO neurons are viable, relying on derepression of autophagy for survival (334).

Autophagy is a process of cellular self-digestion that is initiated typically in times of nutrient paucity. During autophagy, cytosolic and organelar components are surrounded by a double membrane autophagosome which fuses with the lysosome, thereby enabling digestion of the autophagosome contents and release of the resulting raw materials. Autophagy is also utilized by the innate immune system to destroy cytosolic pathogens and has been implicated in the pathology of cancer, diabetes, and heart disease. Several reviews on autophagy have recently appeared (160, 174, 342).

The initiation of autophagy is marked by the appearance of forms of microtubule-associated light chain-3 (LC3) which have been covalently modified with phosphatidylethanolamine to form LC3-II. LC3-II is typically detected as a polypeptide migrating faster upon SDS-PAGE than its unmodified counterpart LC3-I. Inasmuch as LC3-II also binds to burgeoning autophagosome membranes, LC3-II usually can be observed as distinct punctae in the cytosols of autophagic cells. Autophagy also coincides with the proteasomal degradation of sequestrome-1 (p62/SQSTM1) which binds autophagic target proteins and LC3-II (160, 174, 342) (FIGURE 7).

Disruption of all three JNK isoforms in neurons results in elevated LC3-II, and a loss of p62/SQSTM1. In addition, the JNKTKO neurons survive in culture longer than wild-type counterparts. This autophagy requires the transcription factor FoxO, which induces expression of Bnip3. Bnip3 promotes autophagy by displacing the autophagic effector Beclin-1 from inactive Bcl-XL complexes (334). This con-

![FIGURE 7. JNKs in the central nervous system block autophagy, as mediated by several processes (association of Bnip3 with Bcl-XL, lipidation of LC3-I to form LC3-II and autophagosome formation, and degradation of the p62 sequeosome protein). Liberation of these autophagic processes enables neuronal survival when all three JNK isoforms are genetically disrupted.)](http://physrev.physiology.org/)
trasts with results reported by Wei and co-workers (315, 316) showing that JNK promotes autophagy by triggering the interaction of Bcl2 with Beclin-1. Interestingly, JNK\textsuperscript{TKO} neurons show elevated cyclin-dependent kinase activity, which appears to be required for the FoxO-dependent induction of Bnip3 (334) (FIGURE 7).

Davis and colleagues (334) were also able to generate viable mice in which all three JNK isoforms were selectively disrupted in the Purkinje cell layer. These neurons were significantly larger than their wild-type counterparts and, like the JNK\textsuperscript{TKO} cultured neurons, manifested elevated autophagosomes, indicative of enhanced autophagy. Thus the JNKs act redundantly to suppress neuronal autophagy and survival in vivo (334).

B. JNKs and p38s in Cardiac Development

1. JNKs may function with the Wnt pathways to modulate heart development

As was noted above (see sect. IIIC2), the Wnts are a family of secreted proteins that recruit receptors of the Frizzled family to control key cell fate decisions. Noncanonical Wnt signaling proceeds through the adapter protein Disheveled to JNK, possibly through the GCK family kinase GCKR (at least in B cells) (263).

Wnt11 is required for the adoption of a cardiac cell fate in Xenopus. Contemporaneously, Wnt11 triggers enhanced phospho-JNK, and inhibition of JNK signaling with a dominant inhibitory JNK1 construct blocks cardiogenesis stimulated by Wnt11. It should be pointed out, however, that disruption of the JNKs singly has no apparent effect on cardiogenesis (41, 70, 217, 235). Moreover, double disruption of the JNKs in various combinations also does not seem to affect early cardiogenesis, despite causing a substantial impairment of brain development (161, 240) (sect. IVA1). Still, all three JNK isoforms are expressed in the heart, and it is conceivable that they all perform redundant functions in cardiogenesis (functions that could be blunted by overexpression of dominant negative JNK). Disruption of all three JNKs in the heart has not been reported.

2. p38s and cardiogenesis

The role(s) of p38s in cardiogenesis is less well studied. There has been some evidence that p38s may function downstream of BMP receptors, but the p38 isoforms and signaling mechanisms remain to be elucidated unambiguously (235, 299). p38 activity has been implicated in the differentiation of p19 cells into cardiomyocyte-like cells; however, this mechanism, as with the studies of BMP signaling, has not been well characterized (53, 235, 299). Targeted disruption of cardiac p38\textalpha, or, indeed, total body disruption of each of the other three p38 isoforms, does not apparently affect normal heart development (94, 235).

3. MEKK3 and cardiovascular development

Gene disruption studies have demonstrated the importance of stress-activated MAPK pathways to cardiovascular development. Thus disruption of mekk3 is lethal, with death occurring at embryonic day 11. mekk3\textemdash mice manifest a striking impairment of blood vessel development and angiogenesis. Of particular note, this reduced blood vessel formation occurs without a loss of expression of either vascular endothelial cell growth factor (VEGF) or VEGF receptors (340). Instead, disruption of mekk3 produces a pronounced, intrinsic defect in the development of embryonic endothelial cells. In the placenta, this, consequently, impairs the development of embryonic, but not maternal blood vessels (340). Given that MEKK3, via p38, activates the transcription factor MEF2C, which is critical to cardiovascular development (340), it is reasonable to propose that MEKK3 signaling to p38 contributes significantly to the regulation of embryonic angiogenesis (340).

Having said this, it is important to note that disruption of MEF mekk3 impairs not only p38 activation, but NF-\kappaB activation as well (120). Accordingly, it is possible that the effects of mekk3 disruption on heart development involve attenuation of NF-\kappaB instead of, or in addition to, MAPKs.

4. JNKs and p38s in cardiovascular disease-confounding results

Numerous studies using pharmacological agents (often of dubious specificity) and ectopic overexpression of dominant negative/constitutively active MAPK signaling components have been performed to determine the functions of JNKs and p38s in conditions such as ischemic reperfusion disease, heart failure, and cardiac hypertrophy. These studies have produced confusing and often confounding results, likely due to the approaches used and the pleiotropic functions of JNKs and p38s in various aspects of cardiac pathology. A recent excellent review on this subject was published in this journal (235), and the reader is referred there for additional information. The results supporting a salutary or pathophysiological functions for JNKs and p38s will need to be addressed with more specific genetic approaches.

A role for JNKs and p38s in inflammatory and vascular diseases (atherosclerosis, transplant vasculopathy, etc.) is anticipated but as yet unproven. Thus, for example, numerous elements implicated in atherogenesis (TLR-2, -4, and -6 and the adapter MyD88, see also sect. IVCl) are also required for JNK and p38 activation (19, 40, 46, 147, 151).

C. MAPK Pathways and Immune Function

1. MAPK pathways in innate immunity and TLR responses

Innate immunity represents the first line of defense against invading microbial pathogens. Much of the innate immune
response is triggered by engagement of PRRs. PRRs include the membrane-associated TLRs as well as cytosolic nucleotide oligomerization domain-leucine-rich repeat (NOD-LRR) and RIG-like helicase (RLH) proteins. These receptors, upon engagement with agonists, recruit a variety of adapter proteins, most notably the adapter proteins myeloid differentiation factor-88 (MyD88) and Toll/IL-1 receptor (TIR)-domain-containing adapter-inducing interferon-β (TRIF), culminating in recruitment of the E3 Ub ligase TRAF6 (see sect. IIDI). PRRs have been the subject of many recent review articles (19, 147, 151, 195).

PRRs are engaged both by PAMPs, molecular moieties present on invading pathogens, and by DAMPs, molecules produced endogenously in times of physiological stress. DAMPs include oxidized LDL (oxLDL) in atherosclerosis and crystalline uric acid (in gout). Specific PAMPs and DAMPs recruit distinct groups of PRRs. Notably, LPS from Gram-negative bacteria recruits TLR4, and bacterial peptidoglycans recruit TLR-2 and -6. TLR-2, -4, and -6 are also recruited by oxLDL (19, 46, 147, 151, 195).

Activation of PRRs by microbial pathogens leads to a fulminant inflammatory response including cytokine production, leukocyte activation, fever, and, in extreme systemic inflammation/infection, edema, shock and disseminated intravascular coagulation. Much of the inflammatory program implemented by PRRs involves three major signaling mechanisms: NF-κB, interferon response factor, and MAPKs (ERK, JNK, and p38) (19, 147, 151, 195). This discussion will be limited to the complex and cell specific functions of MAPKs.

A) TPL-2. Genetic knockout studies of Tpl-2 reveal an important role for this enzyme in innate and acquired immunity, principally in activation of the ERK pathway by PAMPs and TNF. tpl-2−/− mice produce low levels of TNF upon systemic treatment with LPS and are strikingly resistant to LPS-induced systemic toxicity and lethality. Examination of tpl-2−/− macrophages revealed that LPS stimulation failed to activate MEK1 and ERK while JNK and p38 signaling remained intact. Disruption of tpl-2 also impaired LPS-stimulated macrophage TNF release, as well as activation of ERK by TNF itself. The loss of TNF release by LPS-stimulated tpl-2−/− macrophages appears to be due mainly to the loss of the stimulatory effect ofTpl2 on the ERK-dependent transport of TNF mRNA from the nucleus to the cytosol (69) (FIGURE 8).

Whereas macrophage ERK is targeted principally by Tpl-2, the situation in MEFs is more complicated, indicating a cell-specific quality to Tpl-2 function. Thus disruption of tpl-2 in MEFs impairs LPS activation of ERK, JNK (through, respectively, MEK1 and MKK4), and NF-κB. The latter involves the lack of Tpl-2 catalyzed phosphorylation of NF-κB p65 at Ser276, an event that enhances NF-κB transactivation function (52).

Consistent with these findings are studies showing a role for Tpl-2 in innate and acquired immunity. Thus disruption of tpl-2 (or jnk2) strongly attenuates the development of a Crohn’s-like inflammatory bowel pathology in mice expressing a tnf transgene in which the AU-rich element (sect. IVC3) is deleted (resulting in constitutive TNF expression), genetic evidence confirming that Tpl-2 not only regulates TNF production, but is also downstream of TNF in vivo (157) (FIGURE 8).

Tpl-2 also is critical to host defense, a finding consistent with the central role of Tpl-2 in PAMP signaling, a critical initial step in the host defense response. Thus deficiency of tpl-2 impairs interferon (IFN)-γ production and consequent host defense against Toxoplasma gondii. Reconstitution of rag2−/− mice with Tpl-2-deficient T cells recapitulates this defect in host defense, indicating a key role for Tpl-2 in T cell-mediated host defense (313).

Upon antigen-induced maturation, CD4+ T11 cells undergo polarization to either T111 or T112 effector cells. T111 cells secrete IFN-γ and promote a form of B cell Ig class switching that gives rise to Ig isotypes which provoke macrophage phagocytosis. Accordingly, T111 cells play a key role in cell-mediated immunity (the process by which cells that are infected with viruses and other intracellular microbes such as mycobacteria are eliminated). In contrast, T112 cells produce IL-4 and IL-5. IL-4 also triggers B cell class switching.
However, in contrast to IFN-γ, IL-4 promotes the production of Ig isotypes that do not engender phagocytosis (IgE or, in humans, IgG4). IL-5 activates eosinophils. Accordingly, T<sub>H1</sub>2 cells contribute to immune responses that are phagocytosis-independent, to those dependent on IgE, or to those that employ eosinophils. Such responses include allergy and defenses against helminth parasites.

T<sub>H1</sub>1/T<sub>H2</sub> polarization and the consequent type of immune response are influenced in part by the cytokine milieu present at the time of antigen presentation. Thus certain antigen-presenting cells (APCs; most notably macrophages and dendritic cells) produce IL-12. IL-12 promotes polarization along the T<sub>H1</sub>1 trajectory (the type I cytokine immune response), whereas mature T cells (T<sub>H2</sub> cells in particular, which can also function as APCs) produce IL-4, leading to additional differentiation along the T<sub>H2</sub> path.

T<sub>H1</sub>1 cells naturally express more Tpl-2 polypeptide, and disruption of tpl-2, via impairment of ERK activation, biases towards an exaggerated T<sub>H1</sub>2 response. Consistent with this, CD4<sup>+</sup> T cells from tpl2<sup>−/−</sup> mice exhibited a poor induction of T-bet and a failure to induce Stat4 protein—both signs of weakened T cell-dependent ERK activation, in particular, development of T<sub>H1</sub>1 cells (314) (FIGURE 9A). This fits with studies of the JNK knockouts (66, 239), thus suggesting roles for Tpl-2 and ERK as well as JNK in the differentiation of both subsets of polarized T<sub>H1</sub>1 cells (FIGURE 9A and sect. IVB). Interestingly, lack of MEKK1 also biases TCR-stimulated CD4<sup>+</sup> T cells toward Th2 differentiation, probably as a result of deficient JNK1 and itch activation (73).

B) TAK1 AND MEKK3. Several studies indicate that in addition to Tpl-2, TAK1 and MEKK3 are also involved in the responses to PAMPs and that these responses are cell- and stimulus-dependent. Thus TAK1 is required in T lymphocytes for TCR; IL-2, -7, and 15 recruitment of JNK (FIGURE 9B); and in fibroblasts and B-lymphocytes for PAMP and cytokine activation of JNK (253, 257, 303). TAK1 is also key to early hematopoietic stem cell survival and to the prolonged in vivo survival of hepatocytes (287).

Similarly, in mouse embryonic fibroblasts (MEFs), disruption of mekk3 impairs IL-1 and LPS activation of JNK and p38, but not ERK. In MEFs, MEKK3 is crucial to LPS and IL-1 induction of IL-6 and to TLR-8-mediated activation of MEF cell JNK (120, 226). In macrophage (myeloid tumor) cell lines, however, silencing of mekk3 only modestly inhibits its early-onset (<60 min) LPS stimulation of JNK, p38, and ERK, while exerting a more profound effect at time points later than 2 h, coincident with strongly impaired IL-6 and granulocyte-macrophage colony stimulating factor (GM-CSF) induction. Silencing of mekk3 has no effect on induction of tnf (155) (FIGURE 8).

C) MSKs. Not all elements implicated in MAPK signaling contribute positively to inflammatory responses. Thus MSK1 and MSK2 (sect. IID1) appear to be negative regulators of TLR signaling. Thus downstream of p38 and ERK1/2, MSK1/2 are needed to resolve inflammatory responses by restricting the LPS-stimulated production of proinflammatory cytokines. This is achieved by MSK1/2-dependent induction of the MAPK-inactivating phosphatase dual specificity protein phosphatase 1 (DUSP1) and by induction of the anti-inflammatory cytokine IL-10. Accordingly, mice doubly deficient in MSK1 and MSK2 are hypersensitive to LPS-mediated endotoxic shock and manifest exaggerated inflammation in a model of contact eczema (PMA induced). Thus MSK1 and MSK2 have a negative-feedback role in innate immunity (6) (FIGURE 8). Interestingly, in contrast, DUSP1 induction, in dendritic cells, is apparently mediated instead by MK1/2 phosphorylation and activation of Rsk2 and -3 (348) (see sect. IID1).

D) HPK1. HPK1, which is structurally similar to GCK (see sect. IIC2), also appears to exert a negative effect on both innate and acquired immune functions, although it is not clear if all of these processes are mediated by MAPKs. Thus disruption of hpk1 renders dendritic cells superior to their wild-type counterparts. They express higher levels of the T cell costimulatory molecules CD80 and CD86 and produce more proinflammatory cytokines (IL-12, IL-1β, TNF and IL-6). Consistent with this, hpk1<sup>−/−</sup> dendritic cells can more strongly stimulate T-cell proliferation than can hpk1<sup>+/+</sup> dendritic cells. Interestingly, disruption of dendritic cell hpk1 improves the clearance of Lewis lung carcinoma cells by xenograft studies. Exactly how HPK1 negatively regulates dendritic cell function is unclear (4). As noted in section IIC2, transfected HPK1 is a potent activator of JNK and p38; however, these MAPKs are implicated in proinflammatory and immunostimulatory processes.

In lymphocytes, HPK1 also negatively regulates TCR signaling to AP-1. Thus HPK1 associates with and inactivates the adapter protein SH2 domain-containing leukocyte protein of 76 kDa (SLP76). This negative regulation involves HPK1-catalyzed phosphorylation of SLP76 Ser376, which prevents subsequent Tyr phosphorylation of SLP76 and TCR signaling. It is possible that HPK1 performs a similar function in dendritic cells (62, 177).

2. The JNKs are key regulators of T cell maturation, activation and protection from FasL apoptosis

As revealed in three key genetic studies, the JNKs play a complex role in T-cell maturation and function. APCs express antigen in complex with major histocompatibility complex proteins (MHC-I or II), which interacts with the TCR on naive T cells. CD28, engaged in parallel with the TCR initiates a parallel set of responses, the so-called costimulatory response, needed to elicit optimal T-cell activation culminating in the clonal proliferation of mature, naive
FIGURE 9. MAPKs and lymphocyte functions. A: MAPKs generally favor Th1 T cell polarization. JNK activation, Tpl-2-dependent ERK activation, and Lck/Zap70 or GADD45/MEKK4-dependent p38 activation all have been shown through transgenic mouse studies to contribute to Th1 cell polarization. Somewhat less clear is whether or not these MAPKs actively suppress Th2 polarization, or if genetic disruption merely produces a relative excess of Th2 cells. B: T cell receptor (TCR) engagement recruits several parallel MAPK mechanisms. These include Th1 polarization, through JNK2 activation, Lck/Zap70 or GADD45/MEKK4-dependent p38 activation, or Tpl-2-dependent ERK activation. TCR signaling to MEKK1, independently or through JNK, also recruits Itch to suppress Th2 polarization. JNK1 itself has been directly linked to active suppression of Th2 polarization; whether JNK1 itself does this through the MEKK1/Itch mechanism is somewhat unclear, as the JNK isoforms involved in this process have not been identified (see also FIGURE 4C). It is unclear if MEKK1-dependent JNK activation is sufficient to mediate the JNK-dependent Th1 bias, or if other mechanisms are involved. Notably, MEKK3 activation by TCRs contributes to IFN-γ production and pathogen clearance, while IL-2-, -7-, and -15-dependent JNK activation and responses require TAK1. C: MEKK3, along with MEKK2, also suppresses in Th17 and Treg polarization. TCR recruitment of MEKK2/3-dependent ERK activation promotes ERK-dependent phosphorylation of SMADs-2 and -3, which, in turn, prevents activating SMAD2/3 phosphorylation catalyzed by TGF-β receptors. TGF-β receptor signaling, through SMAD2/3 and the co-SMAD4, fosters Th17 and Treg polarization.
CD4+ T cells to CD4+ Th1 cells. This process requires, in part, the production and subsequent action of certain cytokines such as IL-2. The levels of JNK1, JNK2, as well as MKK-4 and -7 are all quite low in mature naive CD4+ T cells, but increase greatly upon CD3/CD28 costimulation. As implied by this behavior, JNK1/2 are largely dispensable for activation of naive T cells to the Th1 state, and T cells doubly deficient in jnk1 and jnk2 show robust proliferation and unimpaired (and perhaps increased) production of IL-2. The essential functions of JNK-1 and -2 are concerned with the subsequent differentiation of the activated CD4+ T cells into the Th1 or Th2 phenotypes. Inactivation of the genes encoding either JNK1 or JNK2 favors the differentiation of the activated T cells toward the Th2 phenotype (37, 66, 67, 239, 278) (FIGURE 9A). Similarly, treatment of patient-derived T-cell clones reactive with a myelin basic protein (MBP) peptide fragment (AAs 83–99) with peptide mutants that enable activation of JNK/p38 but not ERK provokes a Th1-biased response, whereas other MBP(83–99) mutant peptides that enable ERK but not JNK/p38 activation induce Th12 biased differentiation (267).

Although loss of either jnk1 or jnk2 shifts T-cell differentiation toward a Th12 phenotype, the two JNK isoforms promote differentiation toward Th1 by different mechanisms. Thus the jnk2-deficient Th10 cell fails to produce IFN-γ, indicating that Th1 differentiation is impaired. In contrast, jnk1-deficient Th11 cells produce substantial IFN-γ but nevertheless progress to a Th12 phenotype because of a marked overexpression of IL-4, IL-5, IL-10, and IL-13-cytokines, which promote Th12 differentiation. Thus JNK2 is required for Th11 differentiation, and its absence enables progression to Th12 as a default, whereas JNK1 actively represses Th12 differentiation by suppressing expression of the cytokines necessary for this process, and thus deficiency of jnk1 disinhibits Th12 differentiation. The inhibition of Th12 differentiation by JNK1 is likely due to its negative regulation of NFAT and its ability to activate the E3 ubiquitin ligase Itch, which promotes JunB degradation (37, 66, 67, 73, 239, 278, 302) (FIGURE 9A).

Regarding upstream signaling components that recruit JNK to regulate T-cell polarization, as noted in section II, D2 and F2, studies employing transgenic mice expressing a truncated form of MEKK1 in which the kinase domain was deleted (-KD) indicated that MEKK1 was necessary for TCR recruitment of JNK. MEKK1 can, either directly or through JNK, recruit the E3 Ub ligase Itch and regulate JunB and c-Jun stability. MEKK1 kinase function and Itch activity act to regulate negatively the production of IL-6 as well as the Th12 cytokine IL-4 by promoting the ubiquitination and degradation of JunB. In turn, this suppresses Th12 polarization (73). It is unclear if the observed active suppression of Th12 polarization incurred by JNK1 is regulated through the MEKK1/Itch mechanism, per se inasmuch as the specific JNK isoform recruited by MEKK1 in this pathway has not been identified. In contrast, MEKK1-dependent c-Jun degradation attenuates Th12 tolerance and airway inflammation (302).

Similar studies exploiting the -KD MEKK1 mice also suggest a role for MEKK1 in B-cell function. -KD mice show defective germinal center formation, as well as CD40 activation of JNK and p38 and B-cell receptor-stimulated antibody production. MEKK1 deficiency also led to B-cell hyperproliferation after CD40 ligation (88). As with the T-cell studies, however, these results need to be interpreted with some caution inasmuch as the -KD mice retain the MEKK1 noncatalytic region, and this may lead to off target effects.

The ablation of autoreactive cells occurs early in T- and B-cell development. This process is controlled in part by FasL-induced apoptosis. Disruption of mkk4 in mice attenuates the activation of ES cell JNK by anisomycin and heat shock (208). However, disruption of mkk4 is lethal early in embryonic development, likely due to an impairment of hepatogenesis. mkk4−/− embryos die just after the formation of the primitive vasculature, and contemporaneously with the onset of hepatogenesis. MKK4 likely functions to blunt apoptosis in the liver inasmuch as embryonic lethality consequent to disruption of mkk4 is accompanied by substantial hepatocyte apoptosis (102, 228). Accordingly, to enable examination of the role of MKK4 (and, by extension, the JNKs) in immune cell function, rag2−/− ES cell recipients were microinjected with mkk4−/− ES cells to produce chimeric mice with a MKK4-deficient immune system against a wild-type background. The chimeric T cells were remarkably hypersensitive to apoptosis induced by FasL. This suggests that MKK4 and its substrates may function to protect T cells from apoptosis stimulated by FasL (91, 209).

3. MEKK4, GADD45α, and alternative p38 pathway in T-cell differentiation and responses

In contrast to the JNKs, activation of p38 in T cells is not strictly dependent on costimulation, but is activated by TCR, CD28, and cytokines, e.g., IL-12, in what appears to be a cumulative manner (65). As discussed in section II,E, T-cell p38α can be activated by a MAP2K/MAP3K-independent mechanism involving Tyr323 phosphorylation, followed by autophosphorylation at the activating phosphoacceptor motif Thr180/Tyr182. Tyr323 phosphorylation is catalyzed by the Tyr kinase Zap70 and can be blocked by GADD45α. Disruption of gadd45α leads to constitutive elevation of p38 activity accompanied by lupus-like autoimmune disease, suggesting perhaps a key role for this T-cell specific p38 phosphorylation (250, 251).

The physiological relevance of the noncanonical p38 pathway was documented in a recent study wherein a genetic knockin of a mutant p38α (Y323F) selectively prevented in vivo activation of T-cell p38α. This defect had no effect on CD4+ Th1 differentiation, but instead weakened Th11 re-
sponses, most notably the production of IFN-γ (135) [FIGURE 9B].

In contrast to this, earlier studies indicate a more conventional role for GADD45s and MAP2K/MAP3K-dependent p38 activation in T-cell function. As noted in section IIIE, GADD45, especially GADD45β and -γ, may function to interact with and activate MEKK4; however, the function of this mechanism in the DNA damage response is unclear (259, 284, 308). Instead, disruption of mekk4 reduces TCR activation of CD4+ T-cell [p38 (FIGURE 9B). Of note, mekk4−/− T cells do not produce IFN-γ, indicating that Th11 function or differentiation is impaired. Further studies showed that expression of GADD45β or -γ promotes Th11 function (IFN-γ production), but only in mekk4+/+ T cells. This GADD45β or γ-MEKK4 pathway appears to integrate signals from the TCR to influence positively Th11 cell differentiation (35).

It would appear from these two sets of findings that the different isoforms of GADD45 can differentially regulate p38 activation through the Zap70 nonconventional (inhibited by GADD45α), or the more conventional MEKK4 pathway (enhanced by GADD45β and γ), and through this, Th11 differentiation and function (FIGURE 9B).

MKK3 and its p38 targets also contribute to Th11 development by promoting the production of IL-12 (181). IL-12 is produced by macrophages and dendritic cells as part of the type I cytokine, proinflammatory immune response. As mentioned above, this response helps to foster the differentiation and maturation of naive T cells into Th11 cells. Disruption of mkk3 results in mice that are viable and fertile. However, these mice manifest severely impaired macrophage and dendritic cell IL-12 production. In addition, IFN-γ production and antigen-driven differentiation of naive T cells are reduced significantly. Accordingly, deletion of mkk3 attenuates the type I cytokine immune response (181). Pharmacological inhibition of cultured macrophage cell p38 pathway blunts IL-12 gene transcription. This suggests that a significant component of p38’s effect on IL-12 expression is transcriptional.

4. MEKK3 and T cell responsiveness: MEKK2, MEKK3, and the differentiation of Th17 and Treg T cells

A recent study implicates MEKK3 in T-cell responsiveness. Disruption of T lymphocyte mekk3 has no effect on Th11/2 differentiation, but instead substantially reduces TCR recruitment of ERK, JNK, and p38 (consistent with MEKK3’s apparent in vitro biochemical promiscuity, see sect. IIIF2). In addition, under in vitro nonpolarizing conditions (Th11-TCR engagement in the absence of cytokines that lead to effector T cell differentiation), TCR-mediated induction of IFN-γ production (ordinarily a Th11 response in vivo) is also impaired upon disruption of T cell mekk3. In vivo, this leads to attenuated clearance of Listeria monocytogenes-OVA infection (306) (FIGURE 9B).

Th17 and Treg T cells represent additional subpopulations of CD4+ T cells that function in regulating the severity of inflammation and, perhaps, autoimmunity. Th17 cells are thought to be largely proinflammatory. Their differentiation is promoted in part by IL-17 (which is also secreted by Th17 cells) and TGF-β. In contrast, Treg cells are thought to dampen immune responses and suppress autoimmune and pathological inflammatory responses. Treg differentiation is dependent on the transcription factor Foxp3 and, as with Th17 cell differentiation, is also fostered in part by TGF-β.

Combined, whole body disruption of mekk2 and mekk3 is lethal; however, mekk2−/− mice crossed with mekk3Lck−/−Cre− mice enable T cell lineage-specific deletion of MEKK2 and -3. These mice manifest an excess accumulation of both Th17 and Treg cells in lymphoid tissues (spleen). This process is cell-intrinsic, as the excess Th17 and Treg accumulation is maintained even if knockout embryonic stem cells are used to reconstitute rag2-deficient embryos (306).

Mechanistically, TGF-β in vitro can stimulate naive CD4+CD62LhighCD44lowCD25− T cells into Foxp3+ Treg cells. Likewise, in vivo, TGF-β can be used to stimulate the differentiation of naive CD4+ T cells into IL-17-secreting Th17 cells. Both of these processes are substantially enhanced upon contemporaneous disruption of T cell mekk2 and mekk3, and pharmacological inhibition of TGF-β receptor signaling reduces Treg and Th17 differentiation in vivo in mekk2−/− mekk3Lck−/−Cre− mice. This suggests that mekk2−/− mekk3Lck−/−Cre− mice are hyperresponsive to TGF-β (306) (FIGURE 9C).

Clues as to the mechanistic basis of how the combined disruption of mekk2 and mekk3 affects T-cell polarization come from the observation that MEKK2 and MEKK3 can indirectly regulate the intensity of TGF-β signaling, a critical component of both Th17 and Treg differentiation. Thus disruption of mekk2 and mekk3 impairs TCR recruitment of ERK and p38. This, in turn, reduces ERK (but not p38)-dependent, inhibitory phosphorylation of Similar to Mothers Against Decapentaplegic (SMAD)-2 at Thr220, Ser245, Ser250, and Ser255, and phosphorylation of Similar to Mothers Against Decapentaplegic (SMAD)-3 at Thr179, Ser204, Ser208, and Ser215. The SMADs are transcriptional regulators that are direct targets of the TGF-β receptor. Phosphorylation by the receptors (at Ser465 and Ser467 for SMAD2, or Ser423 and Ser425 for SMAD3) activates triggers binding to SMAD4, nuclear translocation and activation of TGF-β target genes. Inhibitory ERK phosphorylation of the SMAD2/3 blocks phosphorylation of the SMAD proteins...
by the TGF-β receptor, thereby reducing the potency of TGF-β receptor signaling (189, 264). Thus attenuation of this phosphorylation upon disruption of mekk2/3 results in enhanced TGF-β receptor signaling in T cells, thereby increasing T_{H}17 and Treg differentiation (306) (FIGURE 8C).

Although MEKK2/3 can influence the level of both proinflammatory T_{H}17 and anti-inflammatory Treg cells, it is likely that the effect on T_{H}17 cells is more dominant. Thus disruption of mekk2/3 in T cells causes a more severe autoimmune response in the multiple sclerosis model experimental autoimmune encephalitis (306).

5. Role of the JNKs and p38s in promoting cytokine release via posttranscriptional regulation of mRNAs encoding proinflammatory proteins

A robust and efficient innate immune response requires the induction of proinflammatory genes, not just increased transcription, but posttranscriptional stabilization of key mRNAs. Accordingly, proinflammatory stimuli such as bacterial LPS can stabilize mRNAs for IL-1 and TNF as well as those encoding secondary cytokines such as IL-6, IL-8, and genes that regulate the biosynthesis of inflammatory lipids (cyclooxygenase-2, COX2). The posttranscriptional induction of these mRNAs is more rapid than de novo transcription, and while some of these cytokine genes are also induced at the transcriptional level, the posttranscriptional induction mechanism appears dominant inasmuch as the appearance of these mRNAs is insensitive to the transcriptional inhibitor actinomycin. This posttranscriptional mRNA stabilization is largely dependent on p38s-α and -β and their target kinase MK2 (87, 112, 159, 233, 234, 265) (sect. II D1), although JNK can also influence the stabilization of certain mRNAs.

We are only beginning to understand the mechanism by which p38s target mRNAs for stabilization. mRNAs that manifest a high rate of turnover include those encoding TNF, IL-1, COX2, IFN-γ, IL-2, IL-3, IL-6, and (in humans) IL-8 frequently. These mRNAs contain in their 3’ untranslated regions (UTRs), so-called AU-rich elements (AREs). AREs are distinguished by the presence of multiple copies of the sequence AUUUUA. If transferred to stable mRNAs, such as that for β-globin, AREs confer in resting cells destabilization of the target mRNA. In addition, AREs enable signal-induced stabilization of target mRNAs (39). Expression of MKK6 enhances the stability of a chimeric mRNA consisting of the coding region for β-globin and a 161-nt 3’ ARE from the IL-8 mRNA. This reaction can be reversed by a kinase-inactive, dominant inhibitory mutant of p38α (327). The simplest interpretation of these findings is that the ectopically expressed MKK6 recruits p38 which, in turn, induces stabilization of AU-rich mRNAs. Consistent with this, a constitutively active mutant of MK2 could trigger ARE-dependent mRNA stabilization. This suggests that p38-activated mRNA stabilization is mediated by MK2, a p38 substrate (80, 237, 327).

Further support for a role for MK2 and, possibly, MK3 in signal-induced posttranscriptional regulation of mRNA comes from genetic knockout studies. Thus disruption of mk2 severely impairs LPS induction of TNF, and mk2+/- mice show a substantial resistance to LPS-induced shock, due in large part to this loss of TNF production (87, 159). While this effect confers protection against chronic, “sterile” inflammation, including collagen-induced arthritis, disruption of mk2 increases susceptibility to microbial infection, due to a significant reduction in host defense responses (e.g., to Listeria monocytogenes infection) (87, 159). This observation suggests that some caution is warranted in targeting pivotal inflammation pathways to treat chronic inflammatory conditions such as arthritis, atherosclerosis, or type 2 diabetes, as this may produce unwanted attenuation of host defense responses.

Surprisingly, disruption of mk3, which is structurally similar to MK2, is much less effective at reducing LPS induction of TNF; and an mk2.mk3 double knockout produces a modest further decrease in LPS induction of TNF beyond that incurred upon disruption of mk2 alone. Of note, MK3 is, for reasons that remain unclear, considerably less enzymatically active (when assayed against Hsp27) than MK2, perhaps accounting for MK3’s reduced impact on TNF production (87, 233, 234).

As a further demonstration of MK2’s dominant role in LPS regulation of TNF production, disruption of mk5 is without effect on LPS-stimulated stabilization of TNF mRNA. Indeed, mk5+/- mice have no clear phenotype. Analysis of endogenous MK5 activation reveals a differential substrate specificity (despite the observation that recombinant MK-2, -3, and -5 have overlapping substrate specificity in vitro) (265).

MK2 promotes to ARE-mediated mRNA stabilization through the phosphorylation of proteins that bind to AREs and regulate the stability of the cognate mRNAs. Thus MK2, in an agonist-stimulated, p38α-dependent manner, phosphorylates at least three proteins linked to the regulation of mRNA stabilization: tristetraprolin (TTP, at Ser52 (57, 112, 185, 238). MK2 promotes ARE-mediated mRNA stabilization through the phosphorylation of proteins that bind to AREs and regulate the stability of the cognate mRNAs. Thus MK2, in an agonist-stimulated, p38α-β-dependent manner, phosphorylates at least three proteins linked to the regulation of mRNA stabilization: tristetraprolin (TTP, at Ser52 and 178), HuR, and heterogeneous nuclear ribonucleoprotein A0 (hnRNP-A0) (57, 112, 185, 238).

Of these, TTP is best understood. TTP binds AREs (notably that of the TNF message) and fosters the deadenylation and degradation of the cognate mRNAs. MK2 phosphorylation blocks TTP association with AREs, perhaps due to phosphorylation-dependent binding of 14-3-3 proteins to TTP. Phosphorylation also triggers TTP destabilization and deg-
radiation. Consistent with this, disruption of ttp leads to pathophysiological increases in constitutive TNF levels (87, 112, 185) (FIGURE 10).

Interestingly, metabolic stress (hypoxia, nutrient withdrawal), in addition to autophagy, leads to the formation of stress granules (SGs). SGs are regions of stalled mRNA translation the formation of which coincides with p38 activation and TTP phosphorylation. By this process, TTP is excluded from SGs, thereby preventing the degradation of mRNAs present in the SGs (87) (FIGURE 10).

The functions of HuR and hnRNP-A0 are less clear. HuR may, in contrast to TTP, stabilize mRNAs by binding, in a p38/MK2-dependent manner. hnRNP-A0 may function in a similar manner inasmuch as its phosphorylation by MK2 increases binding to the TNF message (57, 238) (FIGURE 10).

Although AREs can regulate mRNA stability, this regulation is frequently manifested differentially. Thus, for example, whereas the mRNA for c-fos contains an ARE, the c-fos mRNA remains labile upon stimulation with agents that regulate known ARE binding proteins. Thus it is plausible to suggest that additional cis elements confer signal-induced mRNA stabilization. IL-2 induction is a characteristic feature of T-cell activation. IL-2 release requires TCR (CD3) and CD28 costimulation (278). While transcriptional induction of contributes strongly to IL-2 production, the signal-induced stabilization of IL-2 mRNA further increases the expression, by activated T cells, of IL-2. The JNKs regulate the stabilization of the IL-2 message through a cis element that spans from the 5'-UTR to the beginning of the coding region (30). SB202190 selectively blocks p38α and -β and is structurally similar to SB20358. At high concentrations, however, SB202190 also blocks JNK. Pretreatment of cells either with SB202190 at levels sufficient to blunt JNK activity, or with cyclosporin, which blocks CD3/CD28 activation of JNK (278) (and p38) prevents IL-2 mRNA stabilization. Of note, the concentrations of SB202190 required to inhibit significantly IL-2 mRNA stabilization were considerably higher than those necessary for complete inactivation of p38. Thus it is likely that the drug was affecting JNK. In contrast, concentration of SB202190 needed to reverse IL-2 message stabilization corresponded closely with that required to prevent the exclusively JNK-catalyzed phosphorylation of c-Jun in situ. In further support for a role for the JNKs in mRNA stabilization, expression of a constitutively active mutant of the JNK-specific MKK7 or of MEKK1, but not a constitutively active mutant of the p38-specific MKK6, or the ERK-specific Raf-1, triggered stabilization of the IL-2 mRNA. Likewise a dominant inhibitory MKK7 mutant blocked MEKK1- or CD3/CD28-stimulated stabilization of the IL-2 mRNA (30).

D. Proinflammatory MAPK Pathways and Cancer

The initial observation that JNKs (and later p38s and ERKs) participated in the activation of AP-1, components of which include the c-Jun protooncoprotein, immediately suggested a role in oncogenesis. Indeed, early studies of c-Jun phosphorylation identified c-Jun phosphorylation at Ser63/73 as a key step in Ha-Ras transformation (270).

Subsequent work identifying JNKs as targets for proinflammatory cytokines, and as enzymes linked to apoptosis, suggested that the JNKs were likely to play a complex role in the regulation of gene expression.
the development of cancers. This has proven to be true, with JNKs and their regulators implicated both in pro- and antioncogenic mechanisms.

1. JNKs and cell proliferation

Disruption of jnk1 modestly impairs the in vitro proliferation of MEFs. In contrast, disruption of jnk2 modestly enhances proliferation. Interestingly, contemporaneous disruption of jnk1 and jnk2 significantly impairs cell proliferation, more so than disruption of jnk1 alone. In these cells, JNK1 accounts for most of the observed c-Jun phosphorylation activity (JNK3 is not detectable in MEFs) (294). The more predominant role for JNK1 in proliferation is consistent with this.

The MEF studies were confirmed and extended in studies of the proliferation of hepatocellular carcinoma cells. Thus disruption of jnk1 in these cells impaired proliferation and tumorigenesis after xenotransplantation (121). This evidence supported a straightforward role for JNK1 in tumor development.

2. JNKs and cellular apoptosis

The implication of the JNKs in neuronal apoptosis is of obvious importance to brain development and function (see sect. IVA); however, apoptosis is also key to tumorigenesis. Thus disruption of jnk1 modestly suppresses ultraviolet-C (UV-C)-induced MEF apoptosis, while disruption of jnk2 is without apparent effect. However, contemporaneous disruption of jnk1 and jnk2 almost completely abolishes UV-C-induced MEF apoptosis. The combinatorial effect of the JNKs is apparently independent of p53 inasmuch as p53 and p73 levels are unaffected by jnk1/2 disruption. Instead, JNK1 and JNK2 are together required for UV-C-induced cytochrome c release and mitochondrial apoptosis. Indeed, reintroduction of cytochrome c into the cytosol restores apoptosis in jnk1−/−;jnk2−/− MEFs (294) (FIGURE 11B).

JNK-mediated mitochondrial apoptosis involves recruitment and regulation of members of the breakpoint cluster region (Bcl)-2 family. Bcl-2 itself is antiapoptotic, preventing cytochrome c release. The activity of Bcl-2 is blocked by sequestration, mediated by the Bcl-2 homology region 3 (BH3) domain-only proteins Bax and Bak. The related BH3 proteins Bim and Bmf may also sequester Bcl-2, thereby promoting apoptosis. These proteins can also bind and activate Bax and Bak (172) (FIGURE 11C).

Bim is itself sequestered and inhibited by binding to the dynein light chain (DLC)-1. This binding requires a short motif in Bim containing a consensus JNK phosphorylation motif. Indeed, JNK phosphorylates Bim at Thr56 and Ser58 in this motif, disrupting the Bim-DLC1 interaction and freeing Bim to promote Bax-dependent mitochondrial apoptosis (172) (FIGURE 11C).

3. JNK1 and chemical-induced hepatocellular carcinoma

Studies using knockout mice further support the notion that JNK1 is integral to cell proliferation and tumorigenesis. Thus partial hepatectomy is a well-established model for cell proliferation and organ regeneration. Posthepatectomy regeneration begins with mitosis of adult hepatocytes but ultimately involves all cell types in the liver, with extensive crosscommunication and intercellular signaling. Disruption of jnk1 or treatment with a JNK inhibitor impairs liver regeneration after partial hepatectomy. As to possible mechanisms, RNAi studies using cultured hepatocellular carcinoma cell lines indicate that JNK1 elevates the expression of c-Myc which, in turn, suppresses the induction of the cell cycle inhibitory protein p21 (122) (FIGURE 11A).

Diethyl nitrosamine (DEN) is a hepatotoxin that engenders hepatocyte death followed by compensatory regeneration. Sustained administration at sublethal doses results in multiple cycles of hepatocyte death and regeneration, ultimately accompanied by the development of hepatocellular carcinoma. DEN-induced liver cancer dramatically enhanced upon disruption of liver IKKα, is strikingly attenuated (albeit not eliminated) upon disruption of jnk1, but not jnk2. While this may be due in part to the elevation in JNK activation that occurs consequent to disruption of ikkβ (286), it is important to note, as discussed below, that neither JNK1 nor JNK2 has any hepatocyte-intrinsic effect on hepatocyte proliferation. The global jnk1 knockouts attenuate the DEN-induced HCC by preventing the Kupffer and/or stellate cells from providing key prooncogenic signals. As predicted by JNK depletion from hepatocarcinoma cell lines, DEN-induced, jnk1−/− liver cancers show reduced c-Myc levels and elevated p21. Suppression of p21 is also required for optimal tumorigenesis. Thus JNK1 is clearly key to tumorigenesis in this model (247, 286) (FIGURE 11A).

4. A more complex, combined and interactive role for JNK1 and JNK2 in hepatocellular carcinoma revealed by dual jnk1/jnk2 disruption

Although a global deficiency of jnk1 clearly suppresses DEN-induced hepatocellular carcinoma, recent studies of the combined role of JNK1 and JNK2 comparing hepatocyte-specific and general JNK deletion identifies a more complex role for JNKs in both promoting and reducing hepatocellular carcinoma. Compound disruption of both jnk1 and jnk2 in hepatocytes was achieved by crossing the hepatocyte-specific JNK1 deficient albumin (Alb)-Cre-Jnk1LoxP/LoxP mice with jnk2−/− mice. In contrast to the global disruption of JNK1 in all liver cell types (see sect.
IVD2), double knockout of jnk1 and jnk2 in hepatocytes, despite modestly slowing cell proliferation (BrDU incorporation), did not affect liver regeneration after partial hepatectomy, and did not inhibit DEN-induced hepatocarcinogenesis, but actually enhanced DEN-induced tumor size, without changing overall tumor incidence or number (51). A global disruption of jnk1 and jnk2 in all liver cell types, i.e., in hepatocytes and nonparenchymal cells such as, e.g.,
Kupffer and Stellate cells, was achieved by crossing Mx1-Cre-\textit{jnk1}\textsubscript{loxP/loxP} mice with \textit{jnk2\textendash\textendash\textendash} mice and administering poly-IC. As with the earlier, global deletion of \textit{jnk1}, disruption of both \textit{jnk1} and \textit{jnk2} in hepatocytes and nonparenchymal cells did suppress DEN-induced hepatocellular carcinoma, and the JNK-dependent proliferation involved downregulation of p21 and upregulation of c-Myc (51). The most plausible explanation for the different responses to hepatocyte-specific versus global deletion of \textit{jnk1} on DEN-induced liver carcinogenesis is that the absence of \textit{jnk1} from the nonhepatocyte cell population diminishes their ability to provide to hepatocytes signals necessary for carcinogenic transformation, such as the protumorigenic cytokines IL-6 and TNF. The effects of hepatocyte-selective \textit{jnk1} deficiency on posthepatectomy regeneration and DEN carcinogenesis indicate that, in contrast to MEFs, hepatocyte JNKs contribute little to the regulation of hepatocyte proliferation (51, 121, 247, 248, 252) (FIGURE 11A).

5. \textit{p38\alpha} antagonizes JNK-dependent hepatocarcinogenesis

While JNK-dependent proliferation is an important contributor to hepatocarcinogenesis, \textit{p38\alpha} (as with IKK\beta/NF-\kappaB; Refs. 247, 286) antagonizes this effect. Thus, although global \textit{p38\alpha} deletion is embryonic lethal due to placental insufficiency (1) and impaired lung function (121), MEFs and hematopoietic cells from \textit{p38\alpha\textendash\textendash\textendash} mice show increased proliferation due to sustained JNK activation (121).

As indicated above, \textit{ikk\beta} disruption fosters enhanced DEN-induced hepatocarcinogenesis in part due to dysregulated JNK activation (247, 248). However, \textit{ikk\beta} disruption also leads to increased ROI production. Interestingly, in the DEN model, ROI production is also elevated upon disruption of \textit{p38\alpha}, despite the presence of intact IKK\beta. This elevated ROI production leads to increased IL-1\alpha release which, as for the combined \textit{jnk1/jnk2} disruption, contributes to sustained JNK-dependent proliferation. Thus \textit{p38\alpha}, by suppressing ROI-dependent IL-1\alpha release, blocks hepatocarcinogenesis (248) (FIGURE 11A). The link between ROI and carcinogenesis is noteworthy given the role of ROI-activated ASK1/2 in cancer (discussed below).

6. JNK is required for Ras-dependent tumor formation in lungs

As noted above, the JNKs, especially JNK1, via activation of AP-1-dependent gene expression, are critical to cell proliferation. Compared with \textit{p53\textendash\textendash\textendash} (\textit{Trp53}) MEFs, \textit{Trp53} MEFs with an additional compound deletion of \textit{jnk1/jnk2} show reduced proliferation. Similar results are seen if the \textit{Trp53}, \textit{jnk1/2\textendash\textendash\textendash}depleted cells express an ectopic activated K-Ras. Indeed, JNKs are required for Ras-induced suppression of contact growth inhibition and coincident loss of E-cadherin (26).

This result was extended to in vivo analysis in studies of mice bearing a lung-specific G12D Ras knock-in versus \textit{Kras\textsubscript{G12D}} mice with a conditional JNK1/2 deficiency (\textit{jnk1}\textsubscript{loxP/loxP}\textit{jnk2\textendash\textendash\textendash}). In this instance, lung-specific JNK1 disruption was achieved by nasal instillation of adenoviral Cre. Here, disruption of the JNKs caused a reduction in the number of K-Ras-G12D-driven hyperplastic lung lesions and adenomas (26).

7. MKK4 and cancer-evidence that MKK4 can act as both a tumor suppressor and tumor promoter

Studies of elements upstream of the JNKs and p38s are less well developed; however, there is evidence that MKK4 has a role in cancer. In this regard, it is important to remember that MKK4 is one of two MKKs implicated in JNK activation and three in p38 activation (sect. II E2) and that it, MKK7, MKK3, and MKK6 may respond to different stimuli, in distinct cell types, to produce different responses.

Loss of function mutations in \textit{mkk4} have been identified in ~5% of tumors from several tissues, and a number of studies have highlighted the importance of MKK4 in suppressing prostate and ovarian tumor cell metastasis (305, 325).

On the other hand, ectopic expression of MKK4 in breast cancer cell lines devoid of \textit{mkk4} promotes proliferation, while RNAi silencing of \textit{mkk4} impairs proliferation of \textit{mkk4}-positive breast cancer cells (205, 325). Moreover, skin-specific deletion of \textit{mkk4} renders mice resistant to chemical carcinogen-induced tumorigenesis, a process mediated by JNK-dependent induction of the EGF receptor (78).

8. Differential roles for ASK1 and ASK2 in apoptosis and tumorigenesis

Apoptosis is a critical mechanism that prevents inappropriate cell proliferation and tumorigenesis. In contrast, inflammation can facilitate tumor cell invasion as well as proliferation of the tumor and plays a major role in metastasis. ASK1 and ASK2 are MAP3Ks that recruit the JNKs and p38s equivalently; however, ASK2 is unstable and only functions biochemically as part of a heteromer with ASK1 (see sect. II F3). Using knockout mice, Iriyama et al. (126) showed that ASK2, in cooperation with ASK1, which is required for ASK2 stability, was tumor suppressive, promoting apoptosis in gut epithelial cells. In contrast, ASK1 alone triggered the production, by inflammatory cells, of cytokines that favored proinflammatory tumorigenesis (126). Thus, as with the JNKs, ASK-1 and -2 perform complex functions in tumorigenesis.

ASK2, in contrast to the ubiquitously expressed ASK1, is most prominently expressed in the skin and gut, notably in the skin keratinocyte layer (126, 282). Disruption of
ask2 does not affect ASK1 levels. Using a two-stage 7,12-dimethylbenz[a]anthracene (DMBA)/phorbol ester (PMA) skin tumorigenesis model, Iriyama et al. (126) observed that disruption of ask2 dramatically increased the incidence and frequency of the resulting dermal papillomas. ASK2 is activated by DMBA and can initiate tumorigenesis, but more prominently promotes keratinocyte apoptosis in response to DMBA. In this capacity, ASK2 fosters the removal of keratinocytes irreversibly damaged by DMBA treatment. As noted in section II F3, both ASKs are activated by ROS, and indeed, DMBA triggers ROI formation, which is the basis for consequent ASK2 activation (126).

As expected from initial findings concerning ASK2, disruption of ask1 also attenuates in vivo stability of ASK2 (see also sect. II F3). In line with this, keratinocyte p38 activation by DMBA is also reduced. Particularly surprising, however, was the observation that disruption of ask1 (with consequent degradation of ASK2) or ask1, plus ask2 did not elevate DMBA/PMA-induced tumorigenesis as did disruption of ask2 alone. The reason for this is that ASK1 is required for the induction, in skin, by DMBA/TPA of proinflammatory cytokines, notably TNF and IL-6 (126) (Figure 11D).

Having said this, the proinflammatory role of ASK1 may actually suppress gut tumorigenesis in inflammatory colitis. Colitis triggered by microbial pathogens is linked with an increased incidence of cancer. When ask1−/− mice were treated with dextran sodium sulfate or Citrobacter rodentium to induce colitis, apoptosis of bone marrow cells was reduced, and colitis was greater than controls, as was subsequent colitis-induced tumorigenesis (106, 107).

E. Proinflammatory MAPK Signaling in Metabolic Disease and Type 2 Diabetes

1. General considerations

The interplay between proinflammatory and hormonal signaling mechanisms is becoming more established. This is especially true for type 2 diabetes, a common complication of obesity and the increasingly sedentary lifestyle seen in Western countries. It is evident that inflammatory signaling pathways can often adversely influence insulin sensitivity, glucose tolerance, and overall metabolic control, contributing to the development of insulin resistance and, eventually, type 2 diabetes.

Thus obesity and insulin resistance, which typically progress to type 2 diabetes mellitus, are associated with a chronic inflammation that includes abnormally elevated levels of proinflammatory cytokines (notably, TNF, IL-1, and IL-6). These abnormalities appear to originate in adipose tissue, which can produce substantial levels TNF. The source of these cytokines may be adipose cells themselves. Indeed, a common histological event in burgeoning insulin resistance is the recruitment by adipose cells of circulating myeloid cells. These cells accumulate around stressed or dying adipocytes to produce so-called crownlike structures which, in turn, produce cytokines and other inflammatory mediators that can influence metabolic control in ways that are still poorly understood.

Signaling by free fatty acids (FFAs) as well as ER stress have also been implicated in the pathophysiology of obesity and insulin resistance. Both FFAs and ER stress can activate the JNKs pathway; however, as is discussed below, mechanisms coupling these stimuli to the JNKs are still nebulous. Despite this, strong evidence has emerged that JNK, especially JNK1, possibly in conjunction with other MAPK groups, can directly contribute to insulin resistance (Figure 12A).

2. Cellular and biochemical effects

An appreciation for the role of MAPKs in the development of insulin resistance first came from studies of the effects of inflammatory cytokines such as TNF-α on insulin resistance in 3T3-L1 adipocytes and rodent models and on the proximal elements in the insulin signal transduction pathway, i.e., the insulin receptor and the insulin receptor substrate (IRS; for review, see Ref. 321) proteins (115, 116, 117). TNF-α was found to impair the ability of IRS-1 to associate with the type 1 PI-3 kinase (141) as well as with the insulin receptor (220), concomitant with increased IRS-1 Ser/Thr phosphorylation. The recognition of the central role of the stress-activated MAPKs as effectors of TNF led to an examination of the contribution of these kinases to the negative regulation of insulin signaling (23, 111), with the demonstration that active JNK associates with and phosphorylates IRS1 in cells at several sites including Ser307; moreover, mutation of IRS-1(Ser307) to Ala largely eliminated the ability of to suppress insulin-stimulated IRS-1 Tyr phosphorylation in CHO cells (2, 3). The importance of JNK in the pathogenesis of insulin resistance was strongly supported by the finding that feeding mice a high-fat diet (HFD) results in JNK activation in muscle, fat, and liver; moreover, global knockout of JNK1 protected mice against obesity when fed a HFD and strongly mitigated the consequent insulin resistance through improved insulin signaling (111). Several mechanisms have been proposed for the ability of a HFD to activate JNK apart from the induction of adipose tissue-derived inflammatory cytokines, including PKC activation, the induction of ER stress, or PRR signaling (129, 156, 215, 221). The role of IRS-1 Ser/Thr phosphorylation as a key mediator of insulin resistance has received considerable attention; the initial observations were followed by a series of reports demonstrating a consistent correlation between IRS-1 (Ser307; Ser312 in humans) phosphorylation and the occurrence of insu-
lin resistance in cellular systems and in genetic or HFD-induced models of rodent obesity (2, 3, 84, 116–118). Recently however, a knock-in of an IRS-1 (Ser307Ala) mutation in the mouse established that the elimination of this phosphorylation site is accompanied by worsened insulin resistance and impaired insulin signaling, establishing that Ser307 phosphorylation is, surprisingly, a positive regulatory event (43). Nevertheless, IRS-1 is phosphorylated at multiple Ser/Thr residues in states of insulin resistance, and it remains likely that some combination of these phosphorylations does confer negative regulation (201) (FIGURE 12A).

**FIGURE 12.** JNK1 and metabolic disease. Note how in most instances, JNK1 acts to inhibit functions that themselves prevent insulin resistance. The notable exception is hepatic JNK1. A: cellular mechanisms of JNK-dependent metabolic dysfunction. High-fat diet (HFD) triggers either ER stress or activation of PKC-ζ/θ-dependent recruitment of JNK1. Activated JNK1 phosphorylates the IRS proteins blunting their Tyr phosphorylation, thereby preventing recruitment of PI-3-kinase-dependent metabolic functions and impairing insulin action. B: JNK1 exerts multiple tissue-specific effects to mediate HFD-induced obesity and insulin resistance. See text for details.
3. A prominent role for JNK1 as a negative regulator of insulin sensitivity in obesity: whole body knockout and bone marrow transplantation studies versus tissue-specific knockouts

The effect of jnk1 disruption on weight gain coincides with a general reduced adiposity in all fat depots plus a reduction in adipocyte size. Interestingly, disruption of jnk1 results in no difference in plasma triglyceride, cholesterol, or FFA concentrations, indicating that lipid metabolism, food intake, and absorption are not affected by disruption of jnk1 (111, 215) (FIGURE 12A).

Disruption of jnk1 also improves the balance in the levels of hormones implicated in insulin sensitivity. Thus the ratio of 30-kDa adipocyte complement-related protein (ACRP30) to adiponectin concentration (a measure of the endocrine regulation of fatty acid oxidation) is higher in jnk1–/– mice, while resistin (a hormone implicated in insulin resistance) levels are lower. In line with this, HFD-fed wild-type mice developed mild hyperglycemia, while jnk1–/– mice had significantly lower blood glucose under identical dietary conditions. Moreover, the obese wild-type but not the jnk1–/– mice also developed hyperinsulinemia, and the jnk1–/– mice fared better in intraperitoneal insulin and glucose tolerance tests (111) (FIGURE 12B).

ob/ob mice develop spontaneous obesity and type 2 diabetes due to loss of function mutations in the leptin receptor. Crossing ob/ob mice with jnk1–/– mice to produce combination jnk1–/–/ob/ob significantly reduces the extent of weight gain compared with ob/ob mice. Likewise, jnk1–/–/ob/ob mice also manifest reduced hyperinsulinemia and hyperglycemia. All of the metabolic effects of the jnk1 deletion coincide with a reduced constitutive phosphorylation of IRS1 at Ser307. Thus JNK1, but not JNK2 (the reasons for this difference are unknown), appears to play an important role in the development of obesity-induced insulin resistance, possibly through direct inhibition of IRS recruitment (111).

Exactly what activates JNK1 in response to HFD/obesity is somewhat unclear. Two mechanisms are emerging as reasonable candidates. The first is elevated circulating triglycerides or FFAs acting as direct agonists for pattern recognition receptors. This is discussed below and in more detail in section IV.E3. The second mechanism involves FFAs triggering ER stress (FIGURE 12A).

ER stress is incurred by elevations in circulating lipids, and obesity places stress on the protein synthetic machinery, of which the ER is an integral part. ER stress is marked by phosphorylation and activation of the protein kinase-R-like kinase (PERK) and phosphorylation of its substrate translational initiation factor 2α (eIF2α). Both of these phosphorylation events, as well as phosphorylation of the JNK and c-Jun polypeptides at the activating sites (Ser63/73; sect. IID2) are strikingly elevated in hepatic tissue from obese mice. Not surprisingly, in Fao hepatoma cells, this phosphorylation coincides with decreased IRS1 levels and Tyr phosphorylation as well as phosphorylation and activation of the Akt protein kinase, a major insulin target downstream of PI-3-kinase (111, 215) (FIGURE 12A).

In the effort to better understand how active JNK promotes whole body insulin resistance, a variety of tissue-specific jnk1 and dual jnk1/jnk2 mouse knockouts have been evaluated. The phenotypes exhibited by these models point out the widespread impact of JNK in metabolic regulation as well as the complex adaptive and maladaptive mechanisms that operate in the wake of selective interruption of such a key regulator.

The most straightforward phenotype is exhibited by the adipose tissue-specific deletion of JNK1 (AdT jnk1KO). These mice exhibit essentially normal adipose development and when placed on a HFD, gain weight similarly to their jnk1-replete littermates. Nevertheless, the HFD-fed mice with jnk1-deficient adipose tissue are essentially protected from the development of whole body insulin resistance, as exhibited by insulin tolerance test. This improvement is due entirely to improved insulin action at the liver; muscle glucose uptake showed little change. HFD increases the expression of a variety of proinflammatory adipokines such as TNF and IL-6; the jnk1-deficient adipose tissue exhibited a marked reduction in IL-6 mRNA and IL-6 blood levels were also significantly reduced, whereas expression of TNF and resistin were unaltered by jnk1 deficiency. SOCS3, an inhibitor of insulin receptor and IRS-1 signaling, is strongly induced in liver by a HFD through IL-6, and the livers of the HFD-fed AdT jnk1 knockout mice exhibited markedly reduced levels of SOCS3 and enhanced insulin receptor tyrosine phosphorylation compared with the HFD-fed, jnk1-sufficient littermates. Thus deletion of jnk1 from adipose tissue greatly enhances insulin responsiveness in liver by a hormonal mechanism (241) (FIGURE 12B).

Obesity results in JNK activation in skeletal muscle, the tissue that is the major site of glucose consumption in vivo. As expected, JNK1 inactivation in skeletal muscle is accompanied by an improvement in insulin sensitivity in mice fed a HFD; unexpectedly however, there is no difference in weight gain or improvement in glucose tolerance, despite the protection from muscle insulin resistance and the preserved muscle glucose uptake. jnk1-deficient muscle exhibits markedly reduced lipoprotein lipase, and circulating triglyceride levels are significantly increased in mice with muscle JNK1 deficiency. These mice develop hepatic steatosis and evidence of inflammation in liver and adipose tissue; hepatic glucose output and adipose tissue glucose utilization remain insulin resistant, presumably offsetting the beneficial response of skeletal muscle (243) (FIGURE 12B).
The importance of hepatic insulin resistance in obesity would suggest that liver-specific inactivation of \( jnk1 \) would have a substantial salutary effect. Moreover, expression of a dominant inhibitory JNK1 in the liver of obese diabetic mice does diminish hepatic glucose output, blood sugar, and insulin resistance (268). Surprisingly however, hepatocyte-specific ablation of \( jnk1 \) results in liver steatosis, insulin resistance, and glucose intolerance (242). This discrepancy is unexplained; whether it indicates a role for hepatocyte JNK2 (silencing of \( jnk2 \) in adult mouse liver, subsequent to HFD-induced steatosis, enhances hepatocyte apoptosis; Ref. 268) or perhaps contributions of JNK1 acting in nonparenchymal cells (as occurs in the DEN-liver apoptosis; Ref. 268) is not clear.

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It is notable that whereas the improved insulin sensitivity of the HFD-fed mice with global disruption of \( jnk1 \) is accompanied by a resistance to weight gain, the improvement seen in the HFD-fed adipose-specific \( jnk1 \) knockout occurs despite a weight gain comparable to the control HFD-fed mice. The explanation for the ability of global \( jnk1 \) knockout to limit weight gain on a HFD appears due in part to the impact of CNS \( jnk1 \) deficiency. A HFD is accompanied by increased phosphorylation of hypothalamic JNK, and local administration of a JNK inhibitor reduces food intake and restores sensitivity of insulin-induced inhibition of food intake (61). Two groups (13, 244) reported the phenotype of mice in which \( jnk1 \) was disrupted selectively in the nervous system by crossing floxed-\( jnk1 \) mice with mice bearing a Cre recombinase construct driven by the nestin promoter (Nestin-Cre mice). \( jnk1 \) disruption in the CNS produced mice which, when fed a standard chow diet exhibited a somewhat smaller body length and reduced lean body mass. However, when placed on a HFD, the mice with CNS \( jnk1 \) deficiency exhibited considerably less weight gain, improved insulin sensitivity in most organs and, when assayed by insulin tolerance test, somewhat lower fasting glucose. Glucose tolerance, revealed by glucose tolerance tests, were similar compared with HFD-fed \( jnk1^{+/+} \) controls (13, 244). The protection from adiposity resulting from HFD reflected a slightly lesser food intake, but a markedly increased level of energy expenditure, due largely to an activation of the hypothalamic-pituitary-thyroid axis (Figure 12B). The mice with CNS \( jnk1 \) deficiency showed increased levels of the circulating thyroid hormones \( T_3, T_4, \) and pituitary thyroid stimulating hormone; hypothalamic levels of thyroid hormone releasing hormone (TRH) mRNA were unaltered, but TRH receptor mRNA levels were considerably increased (13, 244). Belgardt et. al. (13) also showed that treatment of the rat pituitary-derived cell line GH4C1 with the (admittedly nonspecific; Refs. 8, 9, 54) JNK inhibitor SP600125 gave a 60% increase in TRH receptor mRNA, whereas inhibitors of ERK or PI3K were without effect.

Other abnormalities in pituitary-hypothalamic function are evident in the mice with CNS \( jnk1 \) deficiency, e.g., somewhat reduced growth hormone and insulin-like growth factor 1 levels. However, the major factor in the protection from obesity is likely the activation of the hypothalamic-pituitary-thyroid axis (13, 244).

On the basis of the data described, inhibition of JNK activity is currently viewed as a reasonable strategy for the treatment of type 2 diabetes, where obesity and insulin resistance are major factors. It will be of considerable interest to determine whether potent, selective JNK inhibitors, administered systemically, affect glucose, lipid, and energy metabolism in humans, and if so, by what mechanisms.

5. Upstream signaling elements coupling HFD and obesity to JNK

Although few studies have emerged to provide insight into the molecular components that recruit JNK in metabolic disease, it is becoming evident that innate immune signaling mechanisms are key to this process.

Using knockout mice, Greenberg and colleagues (221) have shown that disruption of the MAP3K tpl-2, while having no effect on HFD-induced body weight gain or adipose depot mass, substantially improves insulin sensitivity (hyperinsulinemic-euglycemic clamps) and reduces hepatic gluconeogenesis. This improvement coincides with reduced hepatic steatosis and a substantial reduction in inflammatory markers (reduced adipose tissue crownlike structures, reduced hepatic and macrophage cytokine production). This reduction in obesity-induced insulin resistance and inflammation upon \( tpl-2 \) disruption is accompanied by a reduction
in HFD-induced gonadal fat tissue JNK and ERK activation (221). These findings are consistent with the established role of Tpl-2 as an important MAPK regulator recruited by TNF and pattern recognition receptors (Figure 12, A and B).

It is likely, however, that other mechanisms may couple metabolic stress signals to JNK and p38. Thus, in MEFs, addition of FFAs activates JNK by a process that requires MLK3. This process also requires PKC-ζ insofar as pkcζ−/− MEFs fail to display FFA activation of JNK or MLK3. In white and brown adipose tissue, HFD results in JNK and MLK3 activation. Disruption of mlk3 reduces HFD-induced JNK activation in brown but not white adipose tissue, although it is unclear what cells or agonists are recruiting JNK in this instance. Interestingly, disruption of mlk3 also reduces accumulation of fat in brown but not white adipose tissue. Given that MLK3 and MLK2 often manifest overlapping roles, it is possible that these kinases serve redundant functions that would not be apparent upon disruption of mlk3 alone (18, 129).

V. CONCLUDING REMARKS

When we completed the initial version of this review in 2001, many of the potential wiring diagrams for stress-activated MAPK pathways had been dissected at the biochemical level, and there was a clear need to place these pathways into a physiological context to understand how these pathways functioned in normal and pathophysiological circumstances. It has now become apparent that stress-regulated MAPK pathways are critical both to normal physiology and to many diseases for which inflammation plays an important role.

Inflammation is emerging as a central theme not only in diseases caused by microbial pathogens, but in many of the chronic conditions that continue to plague western cultures. Thus elements of TLR signaling have been shown to be required for type 2 diabetes, cancer, autoimmune disorders, and atherosclerosis.

For several of these chronic conditions, a complete picture is missing as to how proximal elements couple to more distal elements to elicit the established pathology. Type 2 diabetes is perhaps the best characterized of these conditions in terms of linking both proximal (TLR, MyD88) and distal (JNK1) elements to the disease as it manifests itself in numerous tissues. We also know that Tpl-2-dependent ERK activation contributes to pathology. However, it is still unclear how TLRs/MyD88 link to JNK1, or the receptor complexes to Tpl-2, in different tissues and how these pathways integrate to bring about the disease phenotype.

For other chronic conditions, roles for MAPKs are not clearly established, despite an unambiguous link to inflammatory pathways that recruit MAPKs. Thus, for example, atherogenesis has been linked to dysfunctional recruitment of TLR-2, -4, and -6, as well as MyD88. Moreover, engagement of these receptors activates JNK and p38. However, we still do not know the required elements needed to link the receptor complexes to MAPKs and trigger atherogenesis. JNK and its upstream elements have been linked both to tumor promotion and suppression; nevertheless, the mechanisms by which these enzymes are activated in different tumor contexts remain nebulous.

Finally, there is a tendency for researchers in a field to believe that their field holds the panacea for all ills. While it is true that stress- and inflammatory pathway-activated MAPKs have been linked to numerous pathologies, caution is warranted when considering these MAPK pathways or, indeed, any pathway, as a therapeutic target. As we have noted, for example, while inhibition of p38/MK2 may prevent chronic inflammation (e.g., arthritis), it can also lead to an enhanced susceptibility to infectious disease. With the advent of improved mouse models, genomic, proteomic, and metabolomic studies, we anticipate considerable progress in these areas.
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