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Activation and Function of the MAPKs and Their Substrates, the MAPK-Activated Protein Kinases

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INTRODUCTION

Mitogen-activated protein kinases (MAPKs) are protein Ser/Thr kinases that convert extracellular stimuli into a wide range of cellular responses. MAPKs are among the most ancient signal transduction pathways and are widely used throughout evolution in many physiological processes (396). All eukaryotic cells possess multiple MAPK pathways, which coordinately regulate gene expression, mitosis, metabolism, motility, survival, apoptosis, and differentiation. In mammals, 14 MAPKs have been characterized into seven groups (Fig. 1). Conventional MAPKs comprise the extracellular signal-regulated kinases 1/2 (ERK1/2), c-Jun amino (N)-terminal kinases 1/2/3 (JNK1/2/3), p38 isoforms (α, β, γ, and δ), and ERK5 (reviewed in references 54, 198, and 264). Atypical MAPKs have nonconforming particularities and comprise ERK3/4, ERK7, and Nemo-like kinase (NLK) (reviewed in reference 71). By far the most extensively studied groups of mammalian MAPKs are the ERK1/2, JNKs, and p38 isoforms, but recent studies have shed some light on the regulation and function of other groups of MAPKs.

Each group of conventional MAPKs is composed of a set of three evolutionarily conserved, sequentially acting kinases: a MAPK, a MAPK kinase (MAPKK), and a MAPKK kinase (MAPKKK) (Fig. 2). The MAPKKKs, which are protein Ser/Thr kinases, are often activated through phosphorylation and/or as a result of their interaction with a small GTP-binding protein of the Ras/Rho family in response to extracellular stimuli. MAPKKK activation leads to the phosphorylation and activation of a MAPKK, which then stimulates MAPK activity through dual phosphorylation on Thr and Tyr residues within a conserved Thr-X-Tyr motif located in the activation loop of the kinase domain subdomain VIII (Fig. 1). Phosphorylation of these residues is essential for enzymatic activities, as was originally demonstrated for ERK2 (288).

Less is known about the exact molecular mechanisms involved in activation of atypical MAPKs, as they do not share many characteristics of conventional MAPKs (71). One determining feature of atypical MAPKs is that these proteins are not organized into classical three-tiered kinase cascades. In addition, the Thr-X-Tyr motif is absent in ERK3/4 and NLK, where a Gly or Glu residue replaces the Tyr. ERK7 contains the motif Thr-Glu-Tyr in its activation loop, but phosphorylation of these residues appears to be catalyzed by ERK7 itself, rather than by an upstream MAPKK. Nevertheless, once activated, conventional and atypical MAPKs phosphorylate target substrates on Ser or Thr followed by a Pro residue, making them Pro-directed kinases, and therefore have limited specificity in their consensus phosphorylation motifs. In addition to

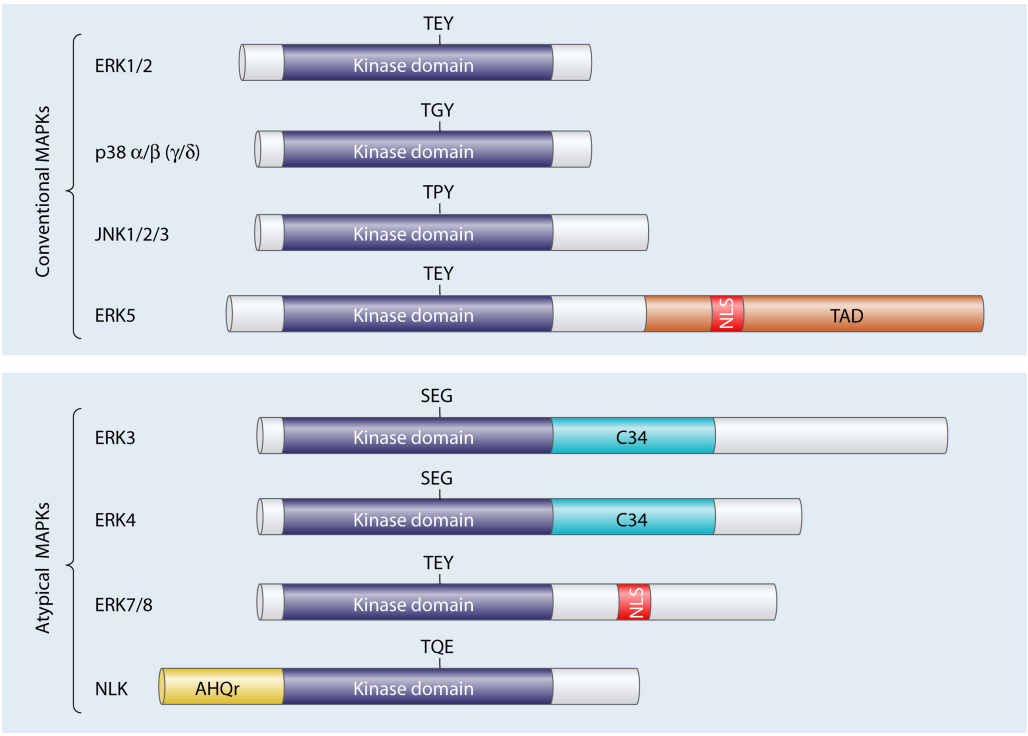

FIG. 1. Schematic representation of the overall structures of conventional and atypical MAPKs. All MAPKs contain a Ser/Thr kinase domain flanked by N- and C-terminal regions of different lengths. Different additional domains are also present in some MAPKs, including a transactivation domain (TAD), a nuclear localization sequence (NLS), a region conserved in ERK3 and ERK4 (C34), and a domain rich in Ala, His, and Glu (AHQr). The respective MAPKAPKs activated by the MAPKs are show in light gray next to the cognate MAPK. The γ and δ isoforms of p38 are in parentheses to indicate that they have not been shown to promote MAPKAPK activation.

the transient kinase-substrate interaction, MAPK substrate selectivity is also conferred by specific interaction domains termed docking sites. Scaffolding proteins also mediate MAPK cascade specificity by simultaneously binding several components and organizing pathways in specific modules.

The wide range of functions regulated by the MAPKs is mediated through phosphorylation of several substrates, including members of a family of protein kinases termed MAPK-activated protein kinases (MAPKAPKs) (Fig. 2) (123, 301). This family comprises the p90 ribosomal S6 kinases (RSKs) (48), mitogen- and stress-activated kinases (MSKs) (14), MAPK-interacting kinases (MNKs) (44), MAPK-activated protein kinase 2/3 (MK2/3) (293), and MK5 (267). MAPKAPK family members represent an additional enzymatic and amplification step in the MAPK catalytic cascades. In addition, they control a wide range of biological functions and thereby increase the range of action regulated by activated MAPK modules. This article gives an overview of the different groups of mammalian MAPKs, as well as describing our current understanding of the properties, regulation, and function of the MAPK-activated protein kinases.

THE CONVENTIONAL MAPKs

The ERK1/2 Module

Identification. ERK1 was the first mammalian MAPK to be cloned and characterized. It was originally found to be phosphorylated on Tyr and Thr residues in response to growth factors (70, 184, 281), and both ERK1 and ERK2 cDNAs were cloned in the early 1990s (35, 36). ERK1 and ERK2 share 83% amino acid identity (Fig. 1) and are expressed to various extents in all tissues, with particularly high levels in the brain, skeletal muscle, thymus, and heart (36). Alternatively spliced isoforms have been described for ERK1 (ERK1b and ERK1c) (325, 418) and ERK2 (ERK2b) (135), and these appear to be activated by specific agonists and may have a different subcellular localization and tissue distribution than full-length proteins (278, 326).

Activation mechanisms and inhibitors. ERK1 and ERK2 are activated by growth factors, including platelet-derived growth factor (PDGF), epidermal growth factor (EGF), and nerve growth factor (NGF), and in response to insulin (36). They are also activated by ligands for heterotrimeric G protein-coupled receptors (GPCRs), cytokines, osmotic stress, and microtubule disorganization (278). The mammalian ERK1/2 module consists of the MAPKKKs A-Raf, B-Raf, and Raf-1, the MAPKKs MEK1 and MEK2, and the MAPKs ERK1 and ERK2 (Fig. 2). While Raf isoforms are the primary MAPKKKs in the ERK1/2 module, the protein kinases MEKK1, Mos, and Tpl2 (also known as Cot) are additional MAPKKKs utilized in a more restricted cell type- and stimulus-specific manner (reviewed in references 278 and 326).

The ERK1/2 module is activated principally by cell surface receptors, such as receptor tyrosine kinases (RTKs). Ligand-induced receptor dimerization promotes receptor activation and autophosphorylation of Tyr residues in the intracellular

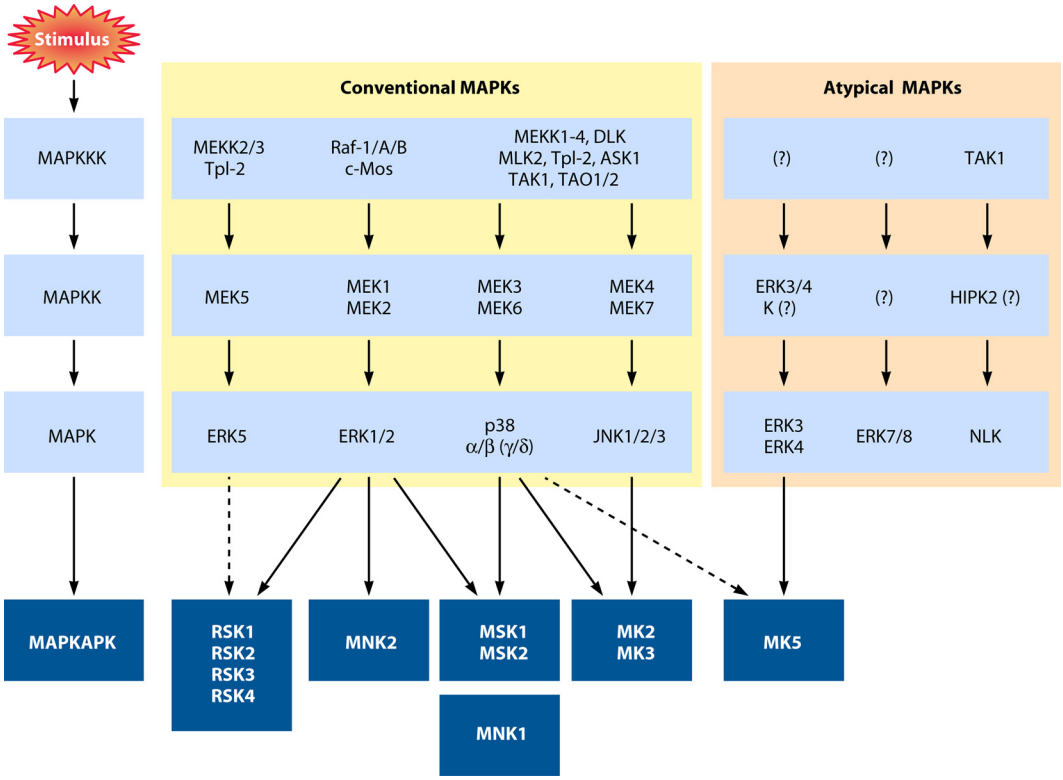

FIG. 2. MAPK signaling cascades leading to activation of the MAPKAPKs. Mitogens, cytokines, and cellular stresses promote the activation of different MAPK pathways, which in turn phosphorylate and activate the five subgroups of MAPKAPKs, including RSK, MSK, MNK, MK2/3, and MK5. Dotted lines indicate that, although reported, substrate regulation by the respective kinase remains to be thoroughly demonstrated. The γ and δ isoforms of p38 are in parentheses to indicate that they have not been shown to promote MAPKAPK activation.

domain. These phosphorylated residues serve as specific binding sites for proteins that contain Src homology 2 (SH2) or phosphotyrosine-binding (PTB) domains, such as Grb2 (growth factor receptor-bound protein 2). The best-characterized route of Ras activation occurs at the plasma membrane and is mediated by SOS (son of sevenless), a guanine nucleotide exchange factor (GEF). SOS is recruited from the cytosol to the plasma membrane as a result of its interaction with Grb2, and it stimulates the exchange of GDP bound to Ras by GTP that is required for a positive regulation of Ras activity. This nucleotide exchange allows Ras to interact directly with its target effectors, one of which is Raf, the initiating kinase of the ERK1/2 module. Regulation of both Ras and Raf is crucial for the proper maintenance of cell proliferation, as activating mutations in these genes lead to oncogenesis (181). Activated Raf binds to and phosphorylates the dual-specificity kinases MEK1/2, which in turn, phosphorylate ERK1/2 within a conserved Thr-Glu-Tyr (TEY) motif in their activation loop (Fig. 1).

MEK1/2 inhibitors have been used extensively to implicate ERK1/2 in a wide array of biological events. Two companies developed MEK1/2 inhibitors in the mid-1990s. One class is typified by PD98059 (8, 98) and the other by U0126 (112). These inhibitors are not competitive with respect to ATP, and they appear to interact with the inactive unphosphorylated kinase more strongly than the active phosphorylated species. This interaction is thought to prevent the phosphorylation of MEK1/2 and/or the conformational transition that generates the activated enzyme (8). More recently, additional noncompetitive inhibitors of MEK1/2 with greater bioavailability (PD184352 and PD0325901) have been developed and entered clinical trials as potential anticancer agents (116). These compounds were tested in a recent study against a panel of recombinant protein kinases (18), which recommended that PD184352 or PD0325901 be used to inhibit MEK1/2 in cells and that the structurally unrelated compound U0126 be used to verify the results.

Substrates and biological functions. In quiescent cells, all components of the ERK1/2 module have a cytoplasmic localization, but upon extracellular stimulation, a significant proportion of ERK1/2 accumulates in the nucleus (53, 213). While the mechanisms involved in nuclear accumulation of ERK1/2 remain incompletely understood, nuclear retention, dimerization, phosphorylation, and release from cytoplasmic anchors have been shown to play a role (reviewed in reference 272). More recently, a new nuclear translocation mechanism for ERK1/2 was identified and is based on a novel nuclear translocation sequence (NTS) located within the kinase insert domain (420). Phosphorylation of this domain upon stimulation allows ERK1/2 to interact with nuclear importing proteins, which mediates the translocation of ERK1/2 into the nucleus via nuclear pores. Upon stimulation, ERK1/2 phosphorylate a large number of substrates (reviewed in reference 416). Some of these are found localized in the cytoplasm (death-associated

protein kinase [DAPK], tuberous sclerosis complex 2 [TSC2], RSK, and MNK) and the nucleus (NF-AT, Elk-1, myocyte enhancer factor 2 [MEF2], c-Fos, c-Myc, and STAT3), whereas others have been found associated with membranes (CD120a, Syk, and calnexin) or the cytoskeleton (neurofilaments and paxillin).

The ERK1/2 module plays a central role in the control of cell proliferation. ERK1/2 activity is rapidly stimulated by mitogenic agents, and in normal cells, sustained activation of these kinases is required for efficient G_1- to S-phase progression. ERK1/2 control cell proliferation via several mechanisms, including the induction of positive regulators of the cell cycle (reviewed in reference 235). ERK1/2 phosphorylate and activate the transcription factor Elk-1, which is involved in expression of immediate-early (IE) genes, such as that for c-Fos (132). ERK1/2 stabilize c-Fos protein through direct phosphorylation (245), thereby allowing c-Fos to associate with c-Jun and form transcriptionally active AP-1 complexes (395). AP-1 activity is required for expression of cyclin D1 (327), a protein that interacts with cyclin-dependent kinases (CDKs) and permits G_1/S transition and cell cycle progression. In addition, ERK1/2 extend the MAPK cascade by phosphorylating and activating MAPKAPK family members, including RSKs, MSKs, and MNKs (Fig. 1 and 2). These protein kinases are important regulators of ERK1/2-dependent biological processes and are discussed below in further detail.

The p38 MAPK Module

Identification. Identified simultaneously by three groups in 1994, p38α (also known as CSBP, mHOG1, RK, and SAPK2) is the archetypal member of a second MAPK module that is generally more responsive to stress stimuli (142, 208, 295). p38α is 50% identical to ERK2 and bears significant homology to the product of the budding yeast *hog1* gene, which is activated in response to hyperosmolarity (142, 208, 295). Since identification of p38α, three additional isoforms have been found, i.e., p38β, p38γ, and p38δ (reviewed in reference 76). Whereas p38α and p38β are ubiquitously expressed in cell lines and tissues, p38γ and p38δ have more restricted expression patterns and may have specialized functions (168). Because p38α is generally more highly expressed than p38β, most of the published literature on p38 MAPKs refers to the former.

Activation mechanisms and inhibitors. In mammalian cells, the four p38 isoforms are strongly activated by various environmental stresses and inflammatory cytokines, including oxidative stress, UV irradiation, hypoxia, ischemia, interleukin-1 (IL-1), and tumor necrosis factor alpha (TNF-α) (reviewed in reference 76). TNF-α and IL-1 activate p38 isoforms by promoting the recruitment of TRAF adaptor proteins to the intracellular domains of their cognate receptors (37). TRAF recruitment promotes activation of various MAPKKKs involved in the activation of the p38 isoforms. The p38 isoforms are also activated by GPCRs (134), as well as by the Rho family GTPases Rac and Cdc42 (17).

MKK3 and MKK6 are thought to be the major protein kinases responsible for p38 activation (89, 143, 344), but MKK4 has also been shown to possess some activity toward p38 (233). While MKK6 activates all p38 isoforms, MKK3 is somewhat more selective, as it preferentially phosphorylates the α, δ, and γ isoforms. The specificity in p38 activation is thought to result from the formation of functional complexes between MKK3/6 and different p38 isoforms and from the selective recognition of the activation loop of p38 isoforms by MKK3/6. Activation of the p38 isoforms results from the MKK3/6-catalyzed phosphorylation of a conserved Thr-Gly-Tyr (TGY) motif in their activation loops (Fig. 1). MKK3/6 are activated by a plethora of MAPKKKs, including MEKK1 to -3, MLK2/3, ASK1, Tpl2, TAK1, and TAO1/2 (76).

Most stimuli that activate p38 MAPKs also stimulate JNK isoforms, and many MAPKKKs in the p38 module are shared by the JNK module (Fig. 2). Identification of the anti-inflammatory drug SB203580 and its close relative SB202190 has been exploited in thousands of studies to delineate the functions of p38. These drugs specifically target and inhibit the p38α and p38β isoforms by acting as competitive inhibitors of ATP binding (208). BIRB0796 is a more potent inhibitor of p38α and p38β that inhibits kinase activity by a novel mechanism that indirectly competes with the binding of ATP (283). A recent study in which these inhibitors were tested against a panel of purified protein kinases concluded that all three compounds have suitable potency and selectivity for their use as p38 MAPK inhibitors in cell-based assays (18).

Substrates and biological functions. p38 isoforms are present in the nuclei and cytoplasm of quiescent cells (20) and have been shown to accumulate in the nuclei of cells subjected to certain stresses (277). While the mechanisms involved in the nucleocytoplasmic shuttling of p38 isoforms remain elusive, the MAPK-activated protein kinases MK2, MK3, and MK5 have been shown to play roles as cytoplasmic anchors for these kinases (122). Upon stimulation, p38 isoforms phosphorylate a large number of substrates in many cellular compartments, including the cytoplasm (cPLA2, MNK1/2, MK2/3, HuR, Bax, and Tau) and the nucleus (ATF1/2/6, MEF2, Elk-1, GADD153, Ets1, p53, and MSK1/2) (76).

The p38 module plays a critical role in normal immune and inflammatory responses (reviewed in reference 76). p38 is activated by numerous extracellular mediators of inflammation, including chemoattractants, cytokines, chemokines, and bacterial lipopolysaccharide (LPS). A major function of p38 isoforms is the production of proinflammatory cytokines. p38 can regulate cytokine expression by modulating transcription factors, such as NF-κB (180), or at the mRNA level, by modulating their stability and translation through the regulation of MNK1 (44) and MK2/3 (293). p38α appears to be the main p38 isoform involved in the inflammatory response, as its deletion in epithelial cells was found to reduce proinflammatory gene expression (186).

The p38 MAPKs have also been shown to play roles in cell proliferation and survival. p38α negatively regulates cell cycle progression at both the G_1/S and G_2/M transitions by a number of mechanisms, including downregulation of cyclins and upregulation of CDK inhibitors (365). Although some studies have reported prosurvival functions for p38α, many more have associated p38α activity with the induction of apoptosis by cellular stresses. These effects can be mediated by transcriptional and posttranscriptional mechanisms, which affect death receptors, survival pathways or pro- and antiapoptotic Bcl-2 family proteins (77). In addition, the p38 isoforms can extend the MAPK kinase cascade by phosphorylating and activating

several MAPKAPK family members, including MSKs, MNK1, MK2/3, and MK5 (Fig. 1 and 2). As briefly suggested above, these kinases are important regulators of p38-dependent processes and will be discussed in greater detail.

The JNK Module

Identification. The first JNK (also known as stress-activated protein kinase [SAPK]) family member was originally identified as a cycloheximide-activated MAP-2 kinase and as a kinase activity that could be affinity purified using c-Jun protein bound to beads (153, 199). It was subsequently found that stress stimuli promote JNK phosphorylation on Thr and Tyr residues, much like what had been observed for the ERKs (200, 202). There are three known JNK isoforms, JNK1 to -3 (also known as SAPKγ, SAPKα, and SAPKβ, respectively), which were independently cloned by two groups in the mid-1990s (88, 201). The JNKs are greater than 85% identical to each other and are encoded by three distinct genes giving rise to 10 or more spliced forms ranging in molecular mass from 46 to 55 kDa (88, 140, 201). JNK1 and JNK2 have a broad tissue distribution, whereas JNK3 seems to be localized primarily to neuronal tissues, testis, and cardiac myocytes (30).

Activation mechanisms and inhibitors. Much like the p38 MAPKs, the JNK isoforms are strongly activated in response to various cellular stresses, including heat shock, ionizing radiation, oxidative stress, DNA-damaging agents, cytokines, UV irradiation, DNA and protein synthesis inhibitors, and growth factor deprivation, and to a lesser extent by growth factors, some GPCR ligands, and serum (reviewed in reference 31). Activation of JNK isoforms requires dual phosphorylation on Thr and Tyr residues within a conserved Thr-Pro-Tyr (TPY) motif in their activation loops (Fig. 1). The MAPKKs that catalyze this reaction are known as MKK4 (also known as SEK1) and MKK7, which appear to cooperate in the phosphorylation and activation of the JNKs (206). MKK4/7 are phosphorylated and activated by several MAPKKKs, including MEKK1 to -4, MLK1 to -3, Tpl-2, DLK, TAO1/2, TAK1, and ASK1/2 (Fig. 2) (198, 382).

With regard to JNK inhibitors, two reversible ATP-competitive inhibitors have been widely used in the last decade, namely, SP600125 (also known as JNK inhibitor II) (22) and AS601245 (JNK inhibitor V) (46). However, a recent study which tested the specificity of these compounds concluded that they had poor selectivity when tested against a panel of purified protein kinases (18). A recent wave of new molecules have been described in the literature over the last 2 to 3 years (reviewed in reference 31), most of which appear to specifically target JNK3.

Substrates and biological functions. Like ERK1/2 and p38 MAPKs, a proportion of activated JNKs have been shown to relocalize from the cytoplasm to the nucleus following stimulation (238). The transcription factor c-Jun is a well-described substrate for JNKs, as its phosphorylation on Ser63/73 was found to increase c-Jun-dependent transcription (reviewed in reference 393). Certain studies have shown that there are functional differences between JNK isoforms with regard to the regulation of c-Jun (306), but more recent evidence using chemical genetics indicated that both JNK1 and JNK2 are positive regulators of c-Jun expression as well as cell proliferation (164). Additional transcription factors have been shown to be phosphorylated by JNK, including p53, ATF-2, NF-ATc1, Elk-1, HSF-1, STAT3, c-Myc, and JunB (31, 278), but very little is known about the contribution of each JNK family member to their regulation. While some cytoplasmic targets of JNK are known, the fact that stimulated JNK does not exhibit an exclusive nuclear localization suggests that many other cytoplasmic substrates remain to be identified.

JNK1 and JNK2 have been shown to play important roles in the control of cell proliferation. Through c-Jun, JNK activity promotes AP-1 complex formation and transcription of genes containing AP-1-binding sites, including genes that control the cell cycle, such as cyclin D1 (306). In addition to having been implicated in the differentiation of hematopoietic populations, JNK isoforms play an important role in the apoptotic response to cellular stresses (90). Mouse embryonic fibroblasts (MEFs) isolated from $Jnk1^{-/-}/Jnk2^{-/-}$ knockout mice are resistant to apoptosis induced by DNA-damaging agents and UV irradiation (368). The inactivation of JNK1 and JNK2 was found to inhibit cytochrome *c* release, indicating a role for the JNK module in the intrinsic apoptotic pathway. Unlike ERK1/2 and p38 MAPKs, there are currently no known MAPKAPK family members directly activated by the JNK isoforms.

The ERK5 Module

Identification. The ERK5 (also known as BMK1, for big MAP kinase 1) pathway was identified independently by three groups using different approaches (106, 209, 434). ERK5 is twice the size of other MAPKs (~100 kDa), and its N-terminal half contains a kinase domain similar to that of ERK1/2, with 51% amino acid identity to ERK2. ERK5 has a unique C-terminal half that contains a nuclear localization signal (NLS) and a proline-rich region (Fig. 1). Three spliced forms of ERK5 have been reported, i.e., ERK5a, ERK5b, and ERK5c (411). ERK5 is expressed to various extents in all tissues, with particularly high levels in the brain, thymus, and spleen (412). ERK5 is essential for early embryonic development and is required for normal development of the vascular system as well as cell survival (282, 340, 412).

Activation mechanisms and inhibitors. ERK5 activity is increased in response to growth factors, serum, oxidative stress, and hyperosmolarity (reviewed in reference 383). This is correlated with the dual phosphorylation of Thr and Tyr residues within a conserved Thr-Glu-Tyr (TEY) motif in the activation loop of the kinase domain. MEK5 was identified as the MAPKK that phosphorylates ERK5 on these residues (106, 434), and other known MEKs do not appear to influence ERK5 activity (Fig. 2). MEK5 is phosphorylated and activated by both MEKK2 and MEKK3 in a stimulus- and cell type-dependent manner (383), but the signaling cascade at this level is not entirely specific to ERK5, since MEKK2/3 also stimulate the JNK and p38 MAPK modules. Finally, WNK1 was recently identified as a potential kinase upstream of MEKK2/3 (408). While PD98059 and U0126 were identified as MEK1/2 inhibitors, these molecules also efficiently inhibit MEK5 (175, 239). Interestingly, MEK5 is less sensitive to PD184352, which can be used as a tool to delineate MEK1/2- and MEK5-dependent functions.

Substrates and biological functions. ERK5 localizes to the cytoplasm in resting cells and translocates to the nucleus when coexpressed with activated MEK5 or upon stimulation (383). A number of molecules have been identified as ERK5 substrates, including the myocyte enhancer factor 2 (MEF2) family of transcriptional factors, Sap1a (ETS domain transcription factor), c-Myc, SGK, connexin 43 (Cx43), and Bad (reviewed in references 147 and 383).

At the cellular level, ERK5 was found to regulate cell survival and proliferation via several mechanisms. Similarly to ERK1/2, activated ERK5 promotes G_1/S transition by driving cyclin D1 expression (244). In addition, the regulation of SGK by ERK5 was found to be essential for S-phase entry in response to growth factors (147). Alternatively, ERK5 has been shown to phosphorylate and activate the RSK family of protein kinases (280), thereby increasing this MAPK kinase cascade by one step (Fig. 2).

THE ATYPICAL MAPKs

ERK3/4

Identification. ERK3 was cloned in 1991 by homology screening of a rat cDNA library using a probe derived from ERK1 sequences (35). Subsequent cloning of the human (234, 435) and mouse (372) orthologs helped established that ERK3 possesses a C-terminal extension of 178 amino acids (aa), yielding a protein with a molecular mass of ~100 kDa. The cDNAs of human and rat ERK4 were isolated using a method similar to that used for ERK3 (126, 135). Whereas it was originally described as a 557-aa protein, resequencing of the human cDNA revealed that ERK4 is a 578-aa protein with a molecular mass of ~70 kDa (71). ERK3 and ERK4 have very similar protein structures, and their kinase domains display 73% amino acid identity. ERK3/4 are considered atypical because their activation loop lacks a phosphoacceptor Tyr residue and contains the Ser-Glu-Gly motif (Fig. 1). The precise function of the C-terminal extension found in ERK3/4 remains elusive, but characterization of this region suggests that it plays a role in subcellular targeting (173). The N-terminal region of ERK3, but not ERK4, is involved in the degradation of ERK3 by the ubiquitin-proteasome pathway (72).

Activation mechanisms and inhibitors. The ERK3/4 module remains poorly characterized (Fig. 2). Although the Ser residue in the activation loop of ERK3 is phosphorylated *in vivo* (55, 56, 73, 86), no stimuli have been found to promote ERK3/4 phosphorylation or activity. Although ERK3 was shown to autophosphorylate *in vitro* (55), a kinase activity toward ERK3 has also been partially purified (55, 56), suggesting that a MAPKK for ERK3 and/or ERK4 may exist. At present, there are no known specific inhibitors of ERK3 and ERK4.

Substrates and biological functions. The only known substrate of ERK3/4 is the MAPK-activated protein kinase MK5, which was identified by several groups as a *bona fide* ERK3/4 phosphorylation target (4, 179, 318, 322). While MK5 is also very poorly understood, the activation mechanisms of MK5 and potential biological functions are described below.

Whereas the biological role of ERK4 is presently unknown, ERK3 has been shown to participate in a number of biological functions, including cell proliferation, cell cycle progression, and cell differentiation. Targeted disruption of the *Mapk6* gene (encoding ERK3) leads to intrauterine growth restriction and early neonatal death, suggesting that ERK3 plays an important role during embryogenesis (189). ERK3 has been suggested to be a negative regulator of cell proliferation (73, 173). ERK3 phosphorylation and protein stability were shown to be regulated by Cdk1 and the Cdc14 phosphatase, suggesting that ERK3 function is regulated throughout mitosis (357). ERK3-mediated proliferation arrest may be linked with its putative role in cellular differentiation. Indeed, it was demonstrated that ERK3 expression increases with the differentiation state of neuronal cells as well as myoblasts (35, 73).

ERK7

Identification. ERK7 was cloned in 1999 by PCR amplification from a rat cDNA library using degenerate primers derived from the kinase domain sequence of ERK1 (2). Thereafter, a probe derived from the sequence of ERK7 was used to identify its human ortholog, which was termed ERK8 (3). ERK7 and ERK8 display 69% amino acid identity, which is lower than what is typical for rodent and human orthologs (71). While ERK7 displays approximately 45% amino acid identity with the kinase domain of ERK1, it is considered atypical partly because it contains a C-terminal extension that is not present in conventional MAPKs (Fig. 1). The precise role of this C-terminal extension is unclear, but it has been shown to play roles in the subcellular localization and autoactivation of ERK7 (1, 2). As is the case for ERK3, the N-terminal region of ERK7 promotes its degradation by the ubiquitin-proteasome pathway (197).

Activation mechanisms and inhibitors. The ERK7 module is poorly characterized, as there are currently no known MAPKKs involved in its activation (Fig. 2). Despite this, the activation loop of ERK7/8 is composed of a Thr-Glu-Tyr motif, suggesting that a classical MAPKK may exist. In the case of ERK7, these residues appear to be constitutively phosphorylated, as they are not modulated by classical stimuli of conventional MAPKs (1, 2). Evidence suggests that they are regulated by autophosphorylation or, at the very least, that ERK7 activity is required for their regulation (1, 2). Consistent with this, ERK8 has also been shown to autophosphorylate *in vitro* and *in vivo* on activation loop residues (3, 188). Conversely to the case for ERK7, certain stimuli of conventional MAPKs have been shown to regulate ERK8 phosphorylation, including serum and H_2O_2 (3, 188). In addition, expression of an oncogenic allele of Src promotes kinase-inactive ERK8 phosphorylation at the Thr-Glu-Tyr motif (3), suggesting that an unidentified MAPKK phosphorylates ERK8 in *trans*. At present, there are no known catalytic inhibitors of ERK7/8, complicating the study of these enigmatic kinases.

Substrates and biological functions. While no *in vivo* ERK7 substrates have been identified thus far, a number of proteins have been shown to be phosphorylated by ERK7 *in vitro*, including classical substrates of conventional MAPKs, such as myelin basic protein (MBP), c-Fos, and c-Myc (2). In the case of ERK8, only MBP has been shown to be a productive substrate for this kinase *in vitro* (188). Despite the lack of *bona fide* ERK7/8 substrates, both protein kinases

play important biological functions, notably in the regulation of cell proliferation (2) and in the response to estrogens (152) and glucocorticoids (307). With regard to MAPKAPKs, there is currently no evidence that ERK7 plays a role in their activation.

NLK

Identification. Nemo-like kinase (NLK) was identified in 1994 by PCR using degenerate primers derived from conventional MAPK sequences (39). NLK is the ortholog of Nemo, a protein kinase previously identified in *Drosophila* (60). NLK displays 45% amino acid identity to the kinase domain of ERK2. It is considered atypical because it possesses N- and C-terminal extensions not present in conventional MAPKs and has a single phosphorylatable residue in its activation loop (Fig. 1). The N-terminal extension is not well conserved between NLK orthologs, and its function remains obscure. The C-terminal extension is conserved from worm to human and may contribute to the interaction of NLK with specific substrates (161).

Activation mechanisms and inhibitors. NLK is activated by stimuli of the Wnt pathway, including Wnt-1 and Wnt-5a (176). Several cytokines have also been reported to activate NLK, including IL-6, granulocyte colony-stimulating factor (G-CSF), and transforming growth factor β (TGF-β) (192, 256). The NLK module is incompletely understood, but the MAPKKK TAK-1 has been reported by several groups to promote NLK activation (161, 256, 336). There are currently no known MAPKKs that directly regulate NLK, but its activation loop is composed of a Thr-Gln-Glu motif (Fig. 1). This motif is reminiscent of the Thr-X-Glu sequence also found in the activation loop of Cdk1, and consistent with this, the kinase domain of NLK is 38% identical to that of Cdk1. Potential MAPKKs that could regulate NLK activation include the kinase homeodomain-interacting protein kinase 2 (HIPK2) (176). This protein was shown to phosphorylate NLK *in vitro* and *in vivo*, but at present it is unclear whether HIPK2 phosphorylates the activation loop of NLK or promotes its autophosphorylation. Consistent with the latter, NLK was found by several groups to autophosphorylate *in vitro* (39, 176).

Substrates and biological functions. Several NLK substrates have been identified to date, including transcription factors of the T-cell factor/lymphoid enhancer factor (TCF/LEF) family (161), as well as STAT3 (192). In response to Wnt stimulation, NLK has been shown to regulate the β-catenin pathway positively or negatively (161, 336). In *Caenorhabditis elegans*, NLK activation by TAK-1 promotes phosphorylation of the TCF/LEF transcription factor POP1, a suppressor of β-catenin-dependent transcription (289). Phosphorylation of POP1 by NLK was found to inhibit its activity, thereby promoting Wnt-dependent establishment of the embryo anteroposterior axis. NLK was also shown to directly phosphorylate the proto-oncogene c-Myb and thereby promote its degradation in response to Wnt signaling (176). With regard to MAPKAPKs, there is currently no evidence that NLK plays a role in their activation.

DOCKING INTERACTIONS

MAPK Docking Domains

D domains. MAPK signaling efficiency and specificity can be achieved in part through specialized docking motifs present in components of the cascade. At least two types of docking interactions between MAPKs and their substrates have been identified, activators and inactivating phosphatases, and both require interaction of short linear sequence motifs present within substrates with a complementary pocket or groove on the kinase. The first docking motif involved in MAPK interaction is the D domain (also referred to as the D site, δ domain, or DEJL domain), which consists of a core of basic residues followed by a hydrophobic patch (Lys/Arg-Lys/Arg-Xaa_{2-6}-Φ-X-Φ, where Φ is a hydrophobic residue, such as Leu, Iso or Val) (reviewed in reference 360). MAPK interactions with D domains have been mapped by mutagenesis, hydrogen exchange-mass spectrometry, and X-ray crystallography (324, 358). Although D domains can sometimes be recognized by more than one group of MAPKs, they are thought to increase signaling specificity and efficacy. D domains lie either upstream or downstream of the phosphoacceptor site and are present on many MAPK regulatory proteins and substrates, including MAPKAPKs (reviewed in references 107 and 123).

DEF domains. The second major MAPK docking site, known as the DEF domain (Docking site for ERK, FXFP; also called the F site or DEF site), has been identified in a number of ERK1/2 substrates. DEF domains are generally characterized by a Phe-Xaa-Phe-Pro sequence, where one of the Phe residues can also be a Tyr (111, 163, 245). This domain is typically located between 6 and 20 amino acids C terminal to the phosphoacceptor site. DEF domains are required for efficient binding to ERK1/2 (210) and have been shown to be required for ERK1/2-mediated substrate phosphorylation (329). Although generally described as a docking site found in ERK1/2 substrates, the DEF domain in the transcription factor SAP-1 contributes to efficient phosphorylation by p38α (125). Currently, no DEF domains have been identified in MAPKAPKs.

CD domain. Two groups independently identified a conserved C-terminal common docking (CD) domain outside the catalytic region of ERK, p38, and JNK involved in D domain interactions (304, 358). The CD domain contains acidic and hydrophobic residues, which are necessary for establishing electrostatic and hydrophobic interactions with the positively charged and hydrophobic residues of D domains, respectively (107, 358). The CD domain is prolonged by a specific 2-aa patch which is neutral in ERK1/2 (TT motif) and acidic in p38 isoforms (ED motif), forming a docking groove for their interacting partners. The importance of these docking interactions was nicely demonstrated by ED/TT motif swapping, which rendered ERK2 capable of binding MK3, a normally exclusive p38 substrate (359). It is important to note that the conserved CD domain is dispensable for the interaction of ERK3 and ERK4 with MK5. A recent study demonstrated, using peptide overlay assays, a novel MK5 interaction motif within ERK3/4 that is essential for binding to the C-terminal region of MK5 (5). While MK5 represents the first described ERK3/4 sub-

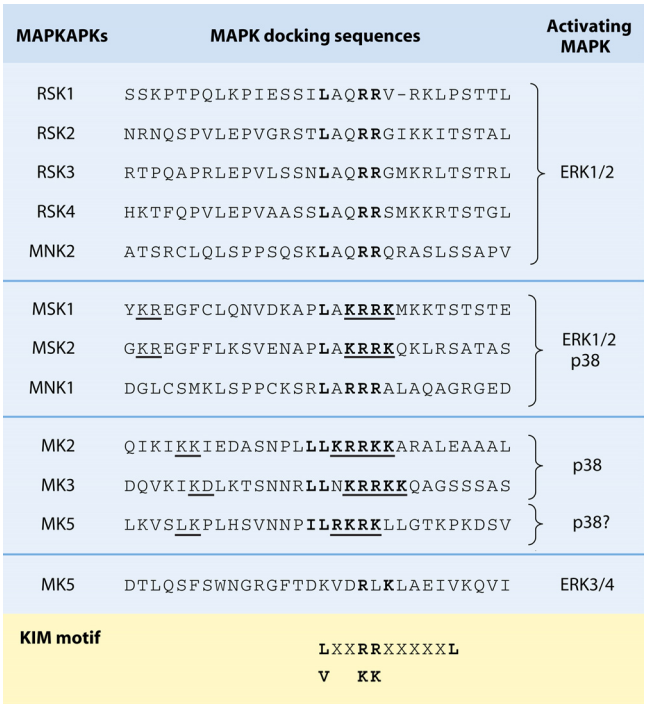

FIG. 3. Alignment of MAPK-binding sequences from MAPKAPKs. MAPKs bind regions termed D domains that are found in all MAPKAPKs and required for efficient activation. The D domains, shown in boldface, are characterized by a stretch of positively charged residues surrounded by hydrophobic residues. Some D domains mediate the specific interaction with ERK1/2 or p38, while others are necessary for the interaction with both upstream activators. In some MAPKAPKs, the D domain region overlaps with an NLS (underlined). ERK3/4 binding to MK5 requires a degenerate D domain in the C-terminal region of the protein. MAPK-binding sequences in MAPKAPKs do not fit the optimal consensus sequence for D domains but rather fit a D domain-like sequence termed the kinase-interacting motif (KIM). Similar alignments were previously demonstrated in recent review articles (adapted from references 123 and 301 with permission).

strate utilizing such an interaction motif, it will be interesting to determine how many more use similar docking mechanisms.

MAPKAPK Docking Motifs

Properties. Except for MK5 binding to ERK3/4, all MAPKAPK family members interact with their cognate MAPKs through their C-terminally located D domains, which do not match the best-characterized consensus sequence for such motifs (360). Instead, D domains found in MAPKAPKs appear to fit the kinase interaction motif (KIM) consensus sequence (224). This D domain-related motif corresponds to a hydrophobic residue closely followed by two positively charged Lys or Arg residues (Leu-Xaa$_2$-Lys/Arg-Lys/Arg-Xaa$_5$-Leu) (Fig. 3). A similar motif has also been reported in several other MAPK substrates, including the tyrosine phosphatase PTP-SL (273) and the cyclic AMP (cAMP)-specific phosphodiesterase PDE4D (224).

MAPK docking specificity arises from variations in D domain sequences, whereby the number of contiguous basic residues appears to correlate with MAPK specificity (338). Generally, ERK1/2-specific MAPKAPKs have two contiguous basic residues, ERK1/2/p38-specific MAPKAPKs have three and four, and p38-specific MAPKAPKs have four and five (Fig. 3). The amount and location of hydrophobic residues within D domains may also regulate specificity, as p38-activated MAPKAPKs have at least two hydrophobic residues before the basic stretch (Fig. 3). Consistent with this, the CD motif in p38 kinases contains more contiguous acidic residues than ERK1/2, suggesting that specific electrostatic interactions between charged residues in CD and D domains provide specificity. Elucidation of the three-dimensional structure of the p38α/MK2 complex (361, 394) demonstrated that a specific Lys residue, present only in MK2 and MK3, specifically interacts with the acidic ED domain of p38α.

As mentioned above, a completely different type of interaction is responsible for MK5 binding to ERK3/4. While the D domain of MK5 has been shown to mediate its interaction with p38, this domain is not required for docking to ERK3/4 (318, 322). A region near the C terminus of MK5 has been shown to mediate binding to ERK3/4, which appears to contain a degenerate D domain sequence (Fig. 3) (4, 179). Additional studies will be required to understand the exact requirements of this atypical interaction region.

Regulation of docking interaction. D domain-mediated interactions are very stable and are often required for preexisting complexes between inactive MAPKs and their substrates. In some cases, MAPK activation regulates docking interactions with MAPKAPKs, a phenomenon that was first described for the interaction between ERK1/2 and RSK family members (302). Indeed, RSK isoforms bind to ERK1/2 in quiescent cells, but following mitogenic stimulation, the complex transiently dissociates for the duration of ERK1/2 activation. Autophosphorylation of a Ser residue near the D domain of RSK was found to promote ERK1/2 dissociation (302), suggesting that a Ser/Thr phosphatase regulates formation of the corresponding inactive complex. Interestingly, MSK isoforms also have autophosphorylation sites near their D domains (14), but whether ERK1/2 or p38 docking is regulated by phosphorylation remains unknown. MNK1 has been shown to interact more strongly with dephosphorylated ERK2 (389), suggesting that MNK1 phosphorylation may also regulate ERK1/2 or p38 docking. Conversely, phosphorylation of ERK3 and ERK4 in their activation loop sites was found to stabilize their interaction with MK5 (86, 266), indicating that MAPKAPK docking interactions are not always weakened upon MAPK activation.

Docking and subcellular localization. Despite having similar general structures, a major difference between RSKs and MSKs is their subcellular localization. While MSK1/2 are constitutively found in the nucleus due to the presence of a bipartite NLS within their MAPK docking sequence, RSK1 to -3 are cytoplasmic enzymes in quiescent cells that translocate to the nucleus upon ERK1/2 stimulation (53). RSK4 does not abide by this rule, as it remains cytoplasmic following most types of stimulation (100). The molecular mechanism involved in RSK translocation remains elusive but likely involves regulated docking to ERK1/2 (302). All MNK isoforms contain a polybasic sequence in their N termini that functions as a potent NLS. MNK1 also contains a functional CRM1-type nuclear export signal (NES) and has been shown to shuttle between the cytoplasm and the nucleus (231, 263). The localization of MNK2 is dictated by alternative splicing, as the long form of

MNK2 (MNK2A) was also found to shuttle between the cytoplasm and the nucleus, whereas the short form (MNK2B) localizes primarily in the nuclear compartment (312). MK2, MK3, and MK5 are much more dependent on their upstream MAPKs for their localization. All three MAPKAPKs display a functional NLS that overlaps with their D domains (Fig. 3), and as a result, expression of p38α was shown to promote the nuclear export of MK3 and MK5 (251, 359). p38-mediated phosphorylation of MK2/3 was shown to regulate their nuclear export through a mechanism that involves unmasking of the C-terminal NES found within these kinases (236, 249). In the case of ERK3/4-dependent regulation of MK5, this interaction was found to promote cytoplasmic accumulation of MK5 in a D domain-independent manner (4, 179, 318, 322).

MAPK-ACTIVATED PROTEIN KINASES

The MAPK-activated protein kinase (MAPKAPK) family contains 11 members (Fig. 4) that are activated by various stimuli depending on their upstream activating kinases (Fig. 1 and 2). Based on homologies within their kinase domains, the MAPKAPKs belong to the calcium/calmodulin-dependent protein kinase (CAMK) family. Of these, the RSK and MSK isoforms contain an additional kinase domain within the same polypeptide, belonging to the AGC (containing PKA, PKG, and PKC families) family of protein kinases (Fig. 4). All MAPKAPK family members share similar activation loop sequences that are targeted for phosphorylation by their cognate upstream MAPKs (Fig. 5A). Based on overall sequence and activation segment homologies, the MAPKAPKs can be classified into five subgroups, the RSKs, MSKs, MNKs, MK2/3, and MK5 (Fig. 5B), which are discussed in greater detail in the following sections.

RSK

Identification and protein structure. RSK was first identified in *Xenopus laevis* extracts (108), and orthologs have since been found throughout metazoans. The human RSK family contains four isoforms (RSK1 [298], RSK2 and RSK3 [174], and RSK4 [299]) that are 73 to 80% identical to each other (Fig. 6). A notable feature of the RSK subfamily of MAPKAPKs is that during evolution, the genes for two distinct protein kinases have fused, generating a single kinase capable of receiving an upstream activating signal from ERK1/2 to the RSK carboxyl-terminal kinase domain (CTKD) and transmitting, with high efficiency and fidelity, an activating input to the RSK amino-terminal kinase domain (NTKD) (Fig. 6). The two kinase domains are distinct and functional (114, 172) and are connected by a regulatory linker region of about 100 aa (Fig. 6). The NTKD belongs to the AGC family of kinases, which also includes protein kinase A (PKA), PKC, Akt, and the S6Ks. The CTKD belongs to the CAMK family, which also includes AMP-activated protein kinase (AMPK), DAPK, and CAMK1/2, and is homologous to the kinase domains found in other MAPKAPKs (Fig. 4). The only known purpose of the CTKD is to activate the NTKD via autophosphorylation (29, 114, 380). In addition, RSK isoforms contain a MAPK docking site that is required for activation by ERK1/2 (127, 338). The D domain in RSK1 to -4 consists of Leu-Ala-Gln-Arg-Arg, where only the Leu and Arg residues are essential for ERK1/2 docking (Fig. 3) (302). Two additional basic residues located C terminal to the D domain also contribute to ERK1/2 docking, but their presence is not essential for RSK1 activation (302).

Tissue expression and subcellular localization. Expression of the RSK1 to -3 mRNAs (but not that of RSK4 mRNA) has been shown to be ubiquitous in every human tissue tested (422). While these results support functional redundancy, some tissue variations have been reported and suggest RSK isoform-specific functions. RSK1 is expressed predominantly in the kidney, lung, and pancreas, whereas both RSK2 and RSK3 are highly expressed in skeletal muscle, heart, and pancreas (7, 241, 422). In the brain, RSK expression is as follows: RSK1 is expressed predominantly in the granular cell layer of the cerebellum; RSK2 in the neocortex, hippocampus, and cerebellum; and RSK3 in the cerebral cortex, the dentate gyrus, and the amygdala (149, 422). RSK4 expression is much lower than those of other RSKs. RSK4 was found to be expressed in the brain, heart, cerebellum, kidney, and skeletal muscle, whereas other tissues, such as lung, liver, pancreas, and adipose tissue, showed no detectable RSK4 expression (100).

During mouse development, RSK2 was found to be expressed at a very low level compared to RSK3, whose mRNA is very abundant in fetal tissues (191). RSK3 expression can be detected in the ventricular zone, a site of high proliferative activity. Conversely, RSK1 expression is strongest in the neuroepithelium of the forming neural tube, whereas at later stages it decreases dramatically and becomes undetectable in the nervous system. These results are consistent with a temporal regulation of RSK1 and RSK3 expression and support the requirement of RSK1 in early and RSK3 in later development of the nervous system. At late stages of development, RSK1 is highly expressed in regions harboring highly proliferating cells. These include liver, lung, thymus, olfactory, and gut epithelia. RSK4 is ubiquitously expressed throughout development but at a very low level in all tissues examined (191). While both the *Rsk1* and *Rsk3* genes give rise to only one transcript, Northern analysis of *Rsk2* expression revealed the alternative use of two different polyadenylation sites giving rise to two transcripts of 3.5 and 8.5 kb (422). Similarly, two secondary *Rsk4* transcripts (5 and 9 kb) also exist, but whether they result from alternative splicing or alternative polyadenylation remains unknown (415).

At the subcellular level, RSK1 to -3 are usually present in the cytoplasm of quiescent cells, but upon stimulation, a significant proportion of these proteins translocates to the nucleus (53, 213, 284, 375, 429). Within minutes of stimulation, RSK1 was shown to accumulate transiently at the plasma membrane, where it presumably receives additional inputs necessary for activation before nuclear translocation (284). RSK4 appears to be predominantly cytoplasmic (100), but in contrast to the case for other RSK isoforms, RSK4 does not significantly accumulate in the nucleus following mitogenic stimulation. The mechanisms involved in RSK translocation to the nucleus remain elusive, but the small death effector domain protein PEA-15 (phosphoprotein enriched in astrocytes 15 kDa) has been shown to interact with RSK2 and inhibit its nuclear translocation (375). Interestingly, RSK2 was recently found to localize to stress granules upon oxidative stress (102), suggesting the possibility that PEA-15 may also localize to these structures. While the mechanisms responsible for the

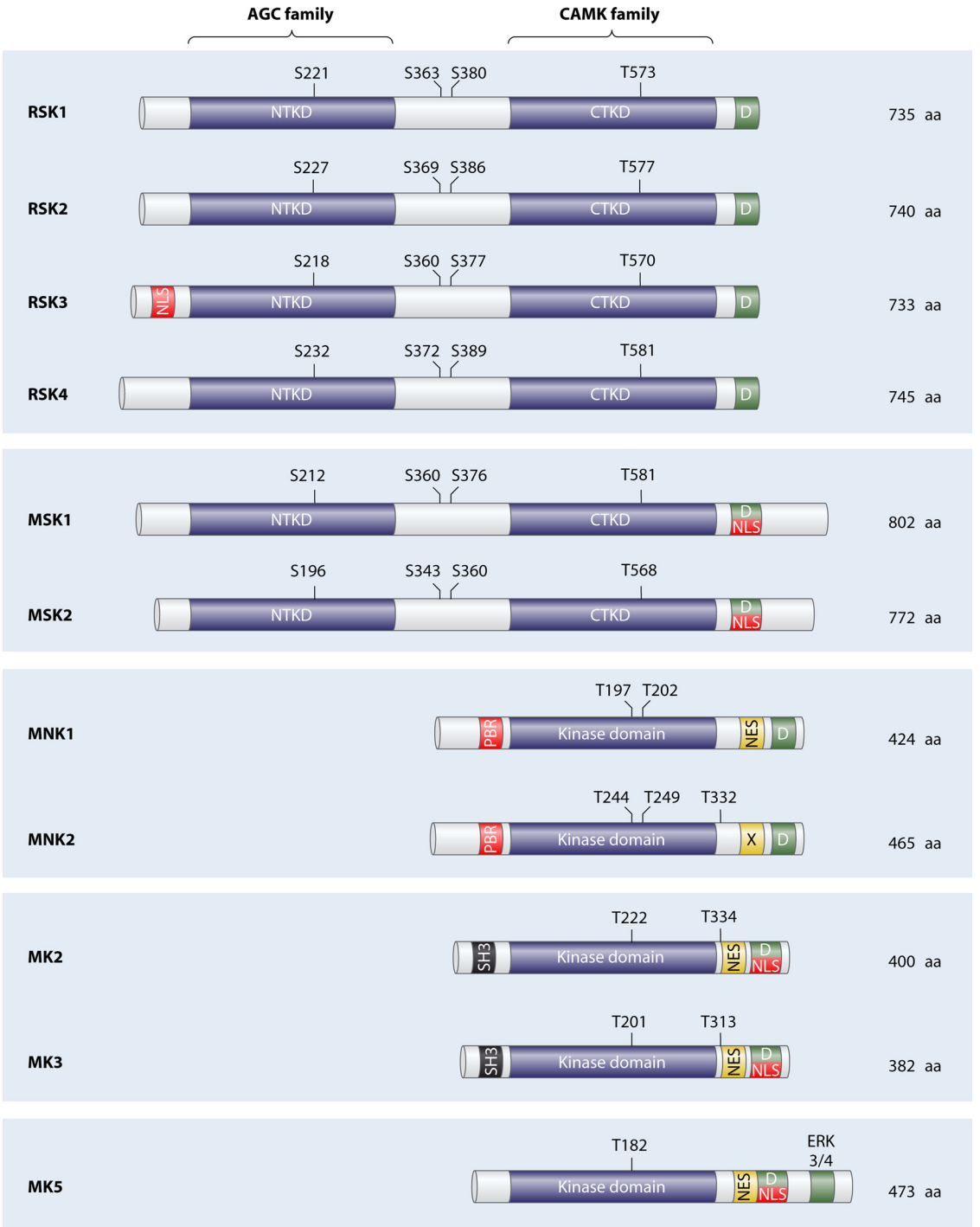

FIG. 4. Schematic representation of the overall structure of the MAPKAPKs. While RSK1/2/3/4 and MSK1/2 are composed of two nonidentical kinase domains, the MNKs and MK2/3/5 are single-headed kinases that display homology to the CTKD of RSKs and MSKs. The NTKDs of the RSKs and MSKs are members of the AGC family of kinases, which also includes Akt, PKA, and PKC. The CTKDs of RSKs and MSKs and the kinase domains of MK2/3/5 belong to the CAMK family of kinases, which also include AMPK, DAPK, and CAMK1/2. The amino acid composition of each MAPKAPK along with phosphorylation site numbering refers to the human nomenclature. Only the full-length form of each MAPKAPK protein is included in this diagram. NES, nuclear export signal; NLS, nuclear localization signal; PBR, polybasic region; SH3, Src homology 3 domain; X, nonfunctional NES; D, D domain or MAPK docking site; ERK3/4, docking region for ERK3/4; NTKD, N-terminal kinase domain; CTKD, C-terminal kinase domain.

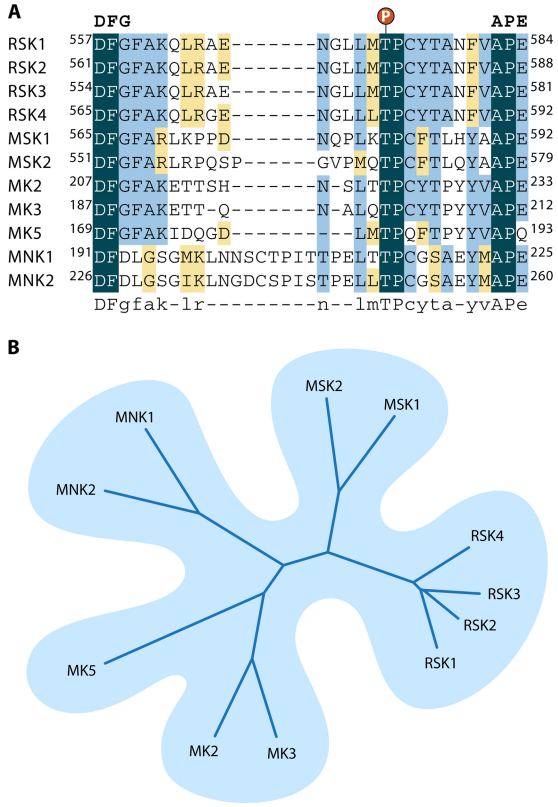

FIG. 5. Sequence analysis of the different MAPKAPKs. (A) Alignment of sequences around activation loops of MAPKAPKs reveal conservation of the MAPK phosphoacceptor residue followed by a Pro. In the case of RSK and MSK, activation loop sequences are from their respective C-terminal kinase domains. (B) Phylogenetic tree of MAPKAPK family members. All sequences used for the construction of this tree were human. The relative similarities between all MAPKAPKs reflected by this tree suggest that the MAPKAPKs are comprised within five groups, the RSK, MSK, MNK, MK2/3, and MK5 subfamilies. The CLUSTAL X program was used to generate the multiple alignments on which the tree was based.

nuclear translocation of the RSK isoforms remain unknown, RSK3 is the only human isoform to possess an optimal NLS, consisting of Lys-Lys-Xaa$_{10}$-Leu-Arg-Arg-Lys-Ser-Arg, but the functionality of this domain has never been tested. Compared to other RSK isoforms, RSK3 is often found associated with insoluble cellular fractions, indicating that RSK3 may localize to specific cellular compartments (P. P. Roux, unpublished data).

Activation mechanisms and inhibitors. RSK activation requires ordered phosphorylation events mediated by ERK1/2, phosphoinositide-dependent protein kinase 1 (PDK1), and RSK autophosphorylation. All RSK isoforms, including *C. elegans* and *Drosophila melanogaster* RSK orthologs, contain the four essential phosphorylation sites (Ser221, Ser363, Ser380, and Thr573 in human RSK1) responsive to mitogenic stimulation (Fig. 4) (78). The current model of RSK activation is that RSK and ERK1/2 form an inactive complex in quiescent cells (156, 428). Upon mitogenic stimulation, ERK1/2 (and probably ERK5) phosphorylate Thr573 located in the activation loop of the CTKD (280, 338, 352) and Thr359/Ser363 in the linker region (78). Activation of the CTKD leads to autophosphorylation at Ser380 located within a hydrophobic motif (380), which creates a docking site for PDK1 (119). For RSK2, this interaction has been shown to increase the catalytic activity of PDK1 by severalfold, indicating that this motif functions to both recruit and activate PDK1. PDK1 is required for mitogenic stimulation of RSK1 to -3, but surprisingly, RSK4 does not appear to require PDK1 to maintain its high basal activity (100). PDK1 association with RSK1 to -3 leads to phosphorylation of Ser221 in the activation loop of the NTKD (167, 285), resulting in full RSK activation (Fig. 7). Recent evidence indicates that RSK2 is also phosphorylated on Tyr residues in response to fibroblast growth factor receptor (FGFR) (177) and Src activation (178). These phosphorylation events were found to stabilize ERK1/2 binding to RSK2 and to promote subsequent activation of RSK2, suggesting an alternative mechanism for RSK activation in human tumors with activated FGFR3 signaling and in response to normal EGF receptor activation.

Mutational inactivation of the CTKD was shown to only partially inhibit activation of the NTKD of RSK1 (62, 302), suggesting that Ser380 phosphorylation may also occur in a CTKD-independent manner (67, 284). Interestingly, the related MK2/3 enzymes were found to phosphorylate Ser380 in certain cell types, which may explain how various stresses that stimulate p38 lead to RSK activation (419). Aside from being involved in RSK phosphorylation, ERK1/2 may also promote RSK1 activation by facilitating its recruitment to the plasma membrane, as suggested by the constitutive activation of a RSK mutant with a myristoylation sequence (284). The process of RSK activation is closely linked to ERK1/2 activity, and MEK1/2 inhibitors (U0126, PD98059, and PD184352) have been used extensively to study RSK function. Recently, three different classes of RSK inhibitors targeting the NTKD (SL-0101 and BI-D1870) or the CTKD (fluoromethyl ketone [FMK]) have been identified (68, 308, 339). While BI-D1870 and SL-0101 are competitive inhibitors with respect to ATP, FMK is an irreversible inhibitor that covalently modifies the CTKDs of RSK1, RSK2, and RSK4. These compounds have been tested against a panel of protein kinases and found to be relatively specific for the RSK isoforms (Fig. 7) (18).

A recent study identified a new point of cross talk between the PKA and ERK1/2 signaling pathways (49). Inactive RSK1 was found to interact with the PKA regulatory I subunit and thereby sensitize PKA to cAMP. However, activation of RSK promotes its interaction with the PKA catalytic subunit, which was found to decrease the ability of cAMP to stimulate PKA. RSK inactivation may require the phosphatase PP2Cδ, which was found to associate with RSK1 to -4 (92). Inactivation of RSK1 may also involve its autophosphorylation at Ser732, which was found to promote ERK/RSK dissociation and correlate with reduced RSK kinase activity (302).

Substrates and biological functions. An important clue about the physiological roles of RSK came from the finding that mutations in the *Rps6ka3* gene (coding for RSK2) were the cause of Coffin-Lowry syndrome (CLS) (Table 1) (370). CLS is an X-linked mental retardation syndrome characterized by psychomotor retardation and facial, hand, and skeletal malformations (145). Numerous mutations have been identified in the *Rps6ka3* gene, most of which result in truncated or inactive RSK2 proteins (85). RSK2 knockout mice have impaired

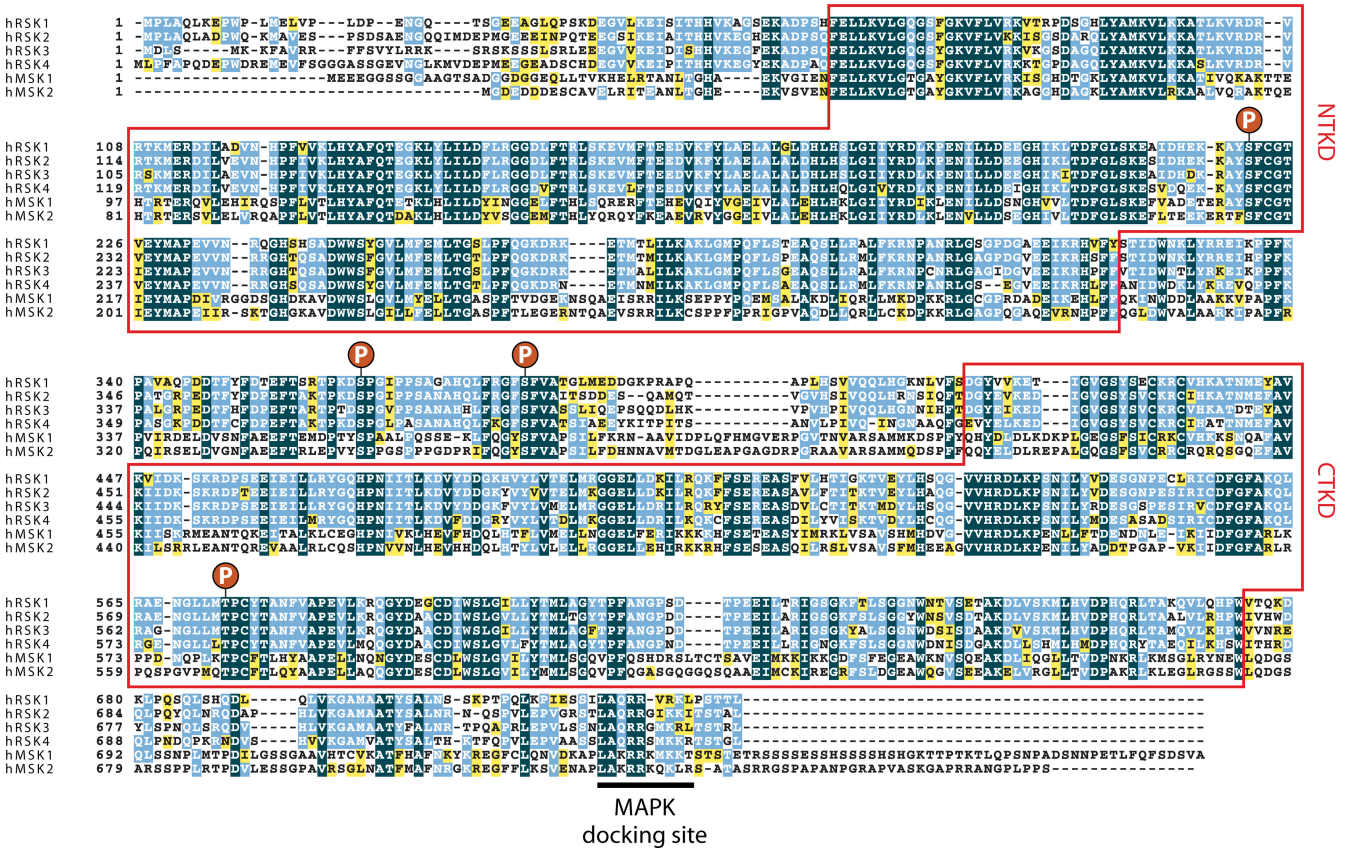

FIG. 6. Alignment of the amino acid sequence of the MAPKAPKs containing two kinase domains. Sequences comprising the kinase domains and its subregions are boxed in red and reveal regions of highest homology. The conserved activation loop threonine residue is shown, as well as other conserved phosphorylation sites. The MAPK-binding domain is identified by a line.

learning and cognitive functions, as well as poor coordination compared to wild-type littermates (99, 271). In addition, they develop a progressive skeletal disease, osteopenia, due to cell-autonomous defects in osteoblast activity (80). Both c-Fos and ATF4 transcription factors have been proposed to be critical targets of RSK2 mediating its effects in osteoblasts (80, 413). RSK2 knockout mice display additional phenotypes. They are approximately 15% smaller than their wild-type littermates, with a specific loss of white adipose tissue that is accompanied by reduced serum levels of the adipocyte-derived peptide leptin (103). RSK1/RSK2/RSK3 triple knockout mice are viable, but no other information regarding their phenotype has yet been reported (101). The *Rps6ka6* gene (which codes for RSK4) is located on chromosome X and was suggested to be involved in nonspecific X-linked mental retardation, but definitive evidence remains to be provided (415). Interestingly, deletion of *Drosophila* RSK was found to result in defects in learning and conditioning (274), but whether these deficits result from the specific loss of RSK activity or deregulated ERK activation or function will require further investigation. Recent evidence has shown that deletion of the *Rps6ka6* gene in the mouse results in early developmental defects, but limited information which would explain how RSK4 regulates embryogenesis was provided (74).

The substrate specificity of RSK1 for target phosphorylation has been determined using synthetic peptide libraries and was found to require the minimum sequences Arg/Lys-Xaa-Arg-Xaa-Xaa-pSer/Thr or Arg-Arg-Xaa-pSer/Thr (212). These data were confirmed using an arrayed positional scanning peptide library, except that results indicated a stronger requirement for Arg residues at positions −3 and −5 than previously thought (P. P. Roux, unpublished data). These analyses also revealed that RSK1 prefers to phosphorylate Ser rather than Thr residues by a factor of at least 5-fold, and consistent with this, the majority of RSK substrates found to date are phosphorylated on Ser residues (Fig. 8).

Although a number of RSK functions can be deduced from the nature of its substrates, data from many groups point toward roles for the RSKs in nuclear signaling, cell cycle progression and cell proliferation, cell growth and protein synthesis, and cell survival. Whereas more substrates have been identified for RSK2 than for any other RSK isoforms, most studies have not determined isoform selectivity. Therefore, many known substrates of RSK2 may be shared by different RSK family members. In addition, many AGC family members share the same consensus phosphorylation motif as RSKs and MSKs, suggesting that substantial overlap in function may exist between RSK and other AGC-related kinases. Indeed, several RSK substrates have been shown to be targeted by Akt, including TSC2, glycogen synthase kinase 3 (GSK3), and $p27^{kip1}$. This also needs to be kept in mind when analyzing phenotypes of knockout mice, as compensation between MK2 and MK3,

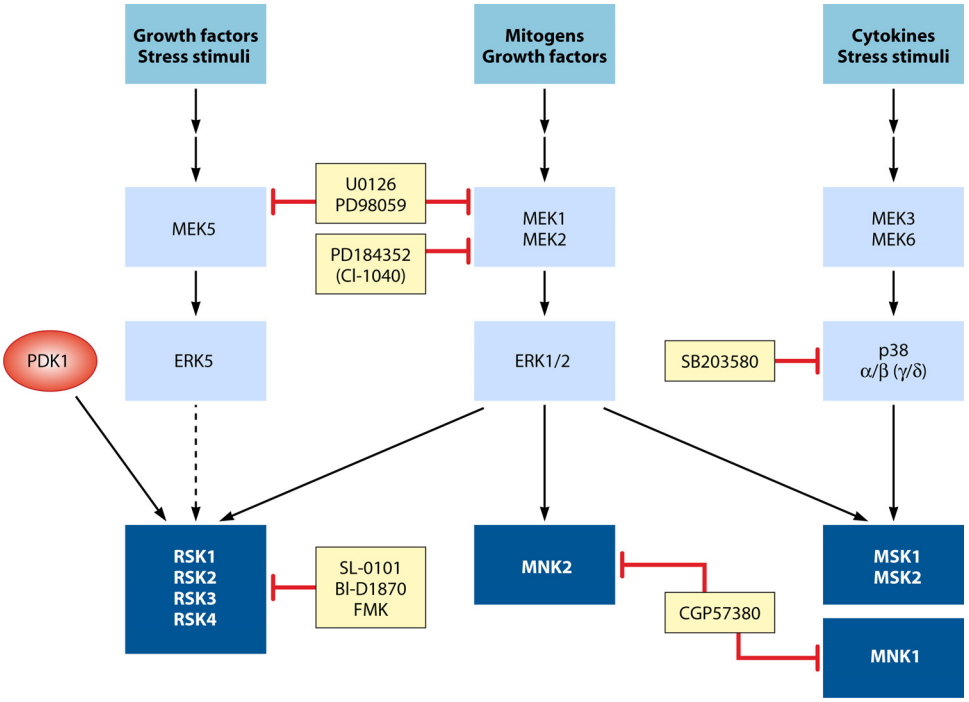

FIG. 7. Signaling cascades leading to activation of the RSKs, MSKs, and MNKs. RSK1/2/3/4 are activated by two inputs originating from ERK1/2 or ERK5, as well as PDK1 enzymes. Similarly, MSK1/2 are activated by ERK1/2 but also by stimuli that activate the p38 module. Depending on the isoforms, MNKs can be stimulated by either ERK1/2 or both ERK1/2 and p38. Different inhibitors of components within these cascades are also shown. Dotted lines indicate that, although reported, substrate regulation by the respective kinase remains to be thoroughly demonstrated.

and probably also other MAPKAPK family members, has been shown to occur.

(i) Nuclear signaling. Activated RSK phosphorylates several transcription factors, some of which contribute to the IE gene response or are IE gene products themselves. Two studies using human cells from CLS patients and primary fibroblasts isolated from *Rsk2*$^{-/-}$ mice showed that RSK2 mediates mitogen-induced *c-Fos* transcription (40, 84). Two distinct mechanisms were proposed, including activation of the Elk1/serum response factor (SRF) complex as well as phosphorylation of the cAMP response element-binding protein (CREB) by RSK2. While CREB was also shown by others to be phosphorylated by RSK2 at Ser133 (133, 407), more recent evidence indicated that the related MSK protein kinases are the predominant CREB kinases operating in somatic cells (398). While the MSKs were shown to be required following mitogenic stimulation

RSK1-4	MSK1/2	MNK1/2	MK2/3	MK5
Gene transcription SRF, Nur77, c-Fos, ER81, TIF-1A, ERα, p65, ATF4 **Cell proliferation** c-Fos, p27^{kip1}, MAD1, LKB1, Myt1, Emi2, GSK3 **Cell growth** rpS6, TSC2, Raptor, eIF4B, LKB1, GSK3, eEF2 kinase **Cell survival** C/EBPβ, DAPK **Others** NHE-1,Filamin A, RanBP3, YB-1, nNOS, AS160	**Gene transcription** CREB, ATF1, p65, ER81, p53 **Nucleosomal response** Histone H3, HMGN1	**mRNA translation** eIF4E, eIF4G **Others** hnRNP A1, PSF, cPLA$_2$, Spry2	**Actin remodeling and cell migration** Hsp25/27, p16-Arc, LSP-1, CapZIP, vimentin, αβ-crystallin **Cytokine production** hnRNP A0, TTP, PABP1, HuR, BRF1 **Gene transcription** BMI1, SRF, ER81, E47 **Cell cycle control** CDC25B, CDC25C, HDM2, TSC2	**Tumor suppression** p53? **Actin remodeling** Hsp25/27?

FIG. 8. Biological functions and substrates of the MAPKAPKs. Upon activation, the RSKs, MSKs, MNKs, and MK2/3/5 phosphorylate several substrates and regulate many biological responses. The list of substrates indicated in this figure is not exhaustive but emphasizes the many important substrates identified to date for the different MAPKAPKs.

TABLE 1. Physiological roles of the MAPKAPKs

MAPKAPK	Gene symbol	Alternate name(s)	Human gene location	Associated disease(s) (reference[s])	Phenotype(s) of knockout mouse (reference[s])
RSK1	*Rps6ka1*	HU-1, MAPKAP-K1A, S6K-alpha 1, p90RSK1	1p36-p35	Expressed at high level in breast and prostate tumors (65, 339)	Viable (101)
RSK2	*Rps6ka3*	HU-3, MAPKAP-K1B, MRX19, S6K-alpha 3, ISPK-1, p90RSK2	Xp22.13	Coffin-Lowry syndrome (370), expressed at a higher level in breast and prostate tumors (65, 339)	Viable and fertile, but knockout animals weigh 10% less than wild-type counterparts (99); reduction in adipose mass, as well as reduced serum leptin levels (103); impaired cognition and coordination skills (271); impaired T-lymphocyte activation (216)
RSK3	*Rps6ka2*	HU-2, MAPKAP-K1C, S6K-alpha 2, p90RSK3	6q27	Potential tumor suppressor in ovarian cancer (28), RSK3 mutations are common in cancer (136)	Viable (101)
RSK4	*Rps6ka6*	S6K-alpha 6, p90RSK4	Xq21	Possible role in nonspecific X-linked mental retardation (415), aberrant expression in breast cancer (362)	Developmental defects (74)
MSK1	*Rps6ka5*	MSPK1, RLPK	14q31-q32.1	None reported	No obvious phenotype (15)
MSK2	*Rps6ka4*	RSK-B	11q11-q13	Located in the locus of Bardet-Biedl syndrome (437)	MSK2 and MSK1/2 double knockouts have no obvious phenotype (398); MSK1/2 play a negative role in innate immune system signaling (10)
MNK1	*Mknk1*		1p34.1	None reported	No obvious phenotype (373)
MNK2	*Mknk2*		19p13.3	None reported	No obvious phenotype (373)
MK2	*Mapkapk2*	MAPKAP-K2	1q32.1	None reported	Knockout mice show increased resistance to endotoxic shock and increased susceptibility to infections (195, 211)
MK3	*Mapkapk3*	MAPKAP-K3, 3pK	3p21.2	Commonly deleted in small cell lung cancer (334)	No obvious phenotype; deletion of MK3 worsens the phenotype of the MK2 knockout mouse (292)
MK5	*Mapkapk5*	MAPKAP-K5, PRAK	12q24.12	None reported	No obvious phenotype (331); MK5-deficient mice are more susceptible to skin cancer (351)

and cellular stresses, some residual CREB phosphorylation was still present in cells derived from *Msk1*$^{-/-}$*Msk2*$^{-/-}$ mice, suggesting that RSK and/or PKA cooperates with MSK1/2 in the phosphorylation of CREB. Related to this, histone H3 was also suggested to be regulated by RSK, but conclusive evidence from the same group demonstrated that MSK1/2 fulfill this function in response to both stress and mitogenic stimulations (341). At the posttranslational level, RSK1 phosphorylates the IE gene products SRF (287), c-Fos (51), and Nur77 (82, 114, 400).

RSK also interacts with the ETS transcription factor ER81 and enhances ER81-dependent transcription by phosphorylating Ser191 and Ser216 (404). ER81 performs many essential functions in homeostasis, signaling response, and development, potentially implicating RSK1 in these processes. The transcription initiator factor TIF-1A also becomes phosphorylated by ERK1/2 and RSK2 following serum stimulation on two Ser residues important for TIF-1A function (427). TIF-1A is required for RNA polymerase I transcription and rRNA synthesis, suggesting that RSK signaling regulates growth-related transcription initiation. The estrogen receptor α (ERα) is an ERK1/2 substrate that becomes activated following stimulation with growth factors. RSK1 associates with and phosphorylates ERα on Ser167, which increases ERα-mediated transcription (170, 410). Another transcription factor, microphthalmia (mi), has been shown to be phosphorylated by the RSKs (406) and has been linked to malignant melanoma (261).

Additional nuclear factors are regulated by RSK, including the NF-κB transcription factor. RSK1 phosphorylates the inhibitory factors IκBα and IκBβ on sites that promote their degradation, thereby stimulating NF-κB activity (131, 317, 409). RSK was also suggested to promote p65 phosphorylation at Ser536 (32, 424), but the role of RSK in phosphorylation of this site remains a subject of debate (94). RSK2 phosphorylates the CREB family member activating factor 4 (ATF4) (413), a transcription factor required for the timely onset of osteoblast differentiation during development. RSK2 was found to be required for osteoblast differentiation and function, suggesting a mechanism by which loss of RSK2 leads to CLS-associated skeletal abnormalities. The transcriptional coactivator CREB-binding protein (CBP) has been identified as a binding target of RSK1 (247). Stimulation of the Ras/MAPK pathway promotes the interaction between RSK1 and CBP (247, 388), but the exact outcome of this association remains to be determined. Interestingly, CBP also interacts with transcription factors that are phosphorylated by RSK1 and RSK2, such as CREB, c-Fos, ER81, ERα, and NF-κB, suggesting that RSK may help in the recruitment of CBP and p300 cofactors to promoters regulated by these transcription factors.

(ii) Cell cycle progression and cell proliferation. Based on the nature of its substrates, RSK is thought to play roles in cell cycle progression and cell proliferation. A recently identified inhibitor of RSK, termed SL-0101 (339), has been tested for its antiproliferative potency. Treatment of cells with SL0101 or RNA interference against RSK1 and RSK2 was found to inhibit proliferation of human prostate and breast cancer cell lines (P. P. Roux, unpublished data) (65, 339). Accordingly, RSK1 and RSK2 were shown to be overexpressed in tumors of the breast and prostate (65, 339), indicating that these two isoforms positively regulate cancer cell proliferation.

The best-characterized molecular mechanism implicating RSK in cell cycle progression involves the regulation of c-Fos, which promotes expression of cyclin D1 during G_1/S transition. RSK2-mediated phosphorylation of c-Fos at Ser362 promotes its stability and oncogenic properties and was found to be essential for osteosarcoma formation in mice (52, 80, 245). RSK1 and RSK2 may also promote G_1-phase progression by phosphorylating the CDK2 inhibitor p27^{kip1} on Thr198 (120, 205). RSK-mediated phosphorylation of p27^{kip1} promotes its association with 14-3-3, which prevents its translocation to the nucleus (120). MAX dimerization protein 1 (MAD1) is a suppressor of Myc-mediated cell proliferation and transformation and was recently identified as an RSK1/2 substrate (436). Phosphorylation by RSK on Ser145 accelerated the degradation of MAD1 by the ubiquitin-proteasome pathway, resulting in increased Myc-dependent transcription. RSK2 phosphorylates the tumor suppressor LKB1, a kinase found mutated in the cancer-prone Peutz-Jeghers syndrome (309). Phosphorylation of LKB1 on Ser431 was not found to affect its activity or membrane association (309), but its phosphorylation was shown to be necessary to prevent LKB1-mediated growth suppression in melanomas through an unknown mechanism (430).

RSK2 appears to be a critical regulator of cell transformation, as ectopic expression of RSK2 was found to increase proliferation as well as anchorage-independent transformation (59). Interestingly, RSK was recently identified as a key effector of Ras/ERK-mediated epithelial-mesenchymal transition (EMT) (93). The transcriptional program initiated by RSK was found to coordinately modulate the extracellular environment, the intracellular motility apparatus, and receptors mediating communication between these compartments to stimulate motility and invasion. In accordance with a role for RSK1 and RSK2 in tumorigenesis, FGFR3 has been recently shown to promote hematopoietic transformation by activating RSK2 in a two-step fashion, by facilitating both ERK-RSK2 interaction and subsequent phosphorylation of RSK2 by ERK (177).

Although RSK3 was initially suggested to play positive roles in cell proliferation (429), this isoform was recently shown to act as a potential tumor suppressor in ovarian cancer (28). RSK3 mutations have been found in a cancer genome sequencing study which evaluated their likelihood of being driver mutations to be very high, providing strong evidence that RSK3 is a tumor suppressor (136). Overexpression of RSK3 was found to reduce proliferation by causing G_1 arrest and increasing apoptosis, but the mechanisms by which RSK3 negatively regulates cell proliferation are currently unknown. One hypothesis is that overexpression of RSK3 may promote a negative feedback loop that suppresses the Ras/ERK cascade (95), but a thorough investigation will be required to fully validate this. The role of RSK4 in proliferation is even more enigmatic. Recent evidence suggests that RSK4 plays an inhibitory role during embryogenesis by negatively regulating RTK signaling (246). RSK4 was also found to participate in p53-dependent cell growth arrest (23) and in oncogene-induced cellular senescence (220), indicating that this isoform behaves somewhat like a tumor suppressor. Consistent with this, exogenous expression of RSK4 resulted in decreased breast cancer cell proliferation and increased accumulation of cells in the G_0/G_1

phase of the cell cycle (363).

The role of RSK in G_2/M progression has been demonstrated by many groups using the preferred model of *Xenopus* oocyte maturation. Immature oocytes are arrested in the G_2 phase of the first meiotic cell division. Addition of progesterone induces the synthesis of the MAPKKK c-Mos, which in turn activates the MEK1-ERK-RSK cascade, leading to M-phase entry and subsequent maturation to an unfertilized egg. M-phase entry is controlled in part by Cdc2, which is a CDK normally kept in check by dual phosphorylation on both Thr14 and Tyr15 by the inhibitory kinase Myt1. RSK2 is the prominent RSK isoform in *Xenopus* oocytes (24), and using this model system, RSK2 was shown to contribute to the control of the meiotic cell cycle at several critical points (316). One mechanism by which RSK2 participates in the progression of oocytes through the G_2/M phase of meiosis I is through phosphorylation and inhibition of the Myt1 kinase (137, 260, 305). It remains unknown whether this mechanism is conserved in other species, but recent efforts demonstrated that Akt can also act as a Myt1 kinase in starfish oocytes (257).

Another way by which RSK can modulate the meiotic cell cycle in *Xenopus* is through ERK-mediated metaphase II arrest, an activity known as cytostatic factor (CSF) (25, 138). RSK1 phosphorylates and activates *in vitro* the kinase Bub1, a mediator of anaphase-promoting complex (APC) inhibition (319), suggesting that RSK1-mediated Bub1 activation contributes, at least in part, to metaphase II arrest (371). Emi2 (also called Erp1) is another APC inhibitor that was initially thought to function independently from the ERK pathway. Three independent studies recently demonstrated that Emi2 is in fact a substrate for RSK. Phosphorylation of Emi2 by RSK promotes Emi2-PP2A association, facilitating Emi2 dephosphorylation at specific Cdc2 phosphorylation sites, which in turn enhances Emi2 stability and function (160, 255, 405).

(iii) Cell growth and protein synthesis. RSK1 was originally identified as an *in vitro* ribosomal protein S6 (rpS6) kinase (108, 109), but S6K1 and S6K2 were later shown to be the predominant rpS6 kinases operating in somatic cells (64). Recent evidence with *S6k1*$^{-/-}$*S6k2*$^{-/-}$ cells (265) and rapamycin-treated cells (303) suggests that the RSK isoforms also contribute to rpS6 phosphorylation *in vivo*. Whereas S6K1/2 phosphorylate all sites on rpS6, RSK1/2 specifically phosphorylate Ser235 and Ser236 in response to Ras/MAPK pathway activation (303). These findings indicated that rpS6 phosphorylation also occurs in an mTOR-independent manner. RSK-mediated rpS6 phosphorylation was found to facilitate assembly of the translation preinitiation complex and to correlate with increased cap-dependent translation (303), providing an additional oncogene- and mitogen-regulated input linking the Ras/MAPK signaling pathway to the regulation of translation initiation.

Ras/MAPK signaling was also found to stimulate mTOR activity through the regulation of the tuberous sclerosis complex (TSC). mTOR is a master regulator of protein synthesis and cell growth, and its activity is controlled by several growth-related pathways. RSK and ERK phosphorylate TSC2 at Ser1798 and Ser664, respectively, which negatively regulates the guanine nucleotide-activating protein (GAP) activity of TSC2 toward the small GTPase Rheb (222, 291, 300). More recently, RSK was shown to phosphorylate Raptor, an important interacting partner of mTOR, providing another link between the Ras/MAPK and mTOR signaling pathways (47). RSK may also regulate mRNA translation through the phosphorylation of GSK3β (11, 353). RSK1-mediated phosphorylation of GSK3β on Ser9 inhibits its kinase activity and thereby releases inhibition on the translation initiation factor eukaryotic initiation factor 2B (eIF2B) (385). Interestingly, activated GSK3β and the LKB1-activated kinase AMPK were both shown to phosphorylate and activate TSC2 (158, 159), suggesting that RSK may inhibit TSC2 activity using direct and indirect mechanisms. Finally, RSK was shown to phosphorylate the eEF2 kinase (386) and the translation initiation factor eIF4B (323), underscoring the involvement of RSK at multiple levels of the pathway leading to protein synthesis.

(iv) Cell survival. RSK1 and RSK2 have been shown to positively regulate cell survival in different cell types. Both RSK isoforms phosphorylate the proapoptotic protein Bad on Ser112, thereby enhancing its ability to bind, and be inactivated by, cytosolic 14-3-3 proteins (34, 332). RSK1 also promotes survival of hepatic stellate cells by phosphorylating C/EBPβ Thr217 in response to the hepatotoxin CCI4 (41). Phosphorylation of Thr217 was suggested to create a functional XEVD caspase inhibitory box that binds and inhibits caspases 1 and 8. More recently, RSK1 and RSK2 were shown to phosphorylate and inactivate death-associated protein kinase (DAPK). Phosphorylation of DAPK at Ser289 inhibits its proapoptotic activity and results in increased cell survival in response to mitogenic stimulation (12). DAPK behaves as a tumor suppressor, and its expression is commonly silenced in tumors through DNA methylation (26). RSK also promotes cell survival through transcription-dependent mechanisms. Indeed, RSK2-mediated phosphorylation of the transcription factor CREB was shown to promote survival of primary neurons through increased transcription of survival-promoting genes, including those for Bcl-2, Bcl-XL, and Mcl-1 (34, 407). More recently, RSK1 was found to promote survival through the activation of the transcription factor NF-κB (131, 317, 423).

(v) Other substrates. In addition to regulating transcriptional and translational programs related to cell growth and proliferation, the RSK isoforms have been shown to phosphorylate many more substrates involved in diverse cellular processes. RSK2 phosphorylates the Na^+/H^+ exchanger isoform 1 (NHE-1) (356), a key member of a family of exchangers that regulate intracellular pH and cell volume (355). RSK2-mediated phosphorylation of NHE-1 on Ser703 was found to regulate mitogen-dependent Na^+/H^+ exchange and intracellular pH (356). RSK2 may also phosphorylate on Ser1152 the cell adhesion molecule L1 (402), a protein that becomes hyperphosphorylated during periods of high neuronal activity, suggesting the involvement of RSK2 in neurite outgrowth. Recently, RSK1 was suggested to play a role in membrane ruffling by phosphorylating the cytoskeleton-associated protein filamin A on Ser2152 (403). Ser2152 was previously shown to be phosphorylated by PAK1 and to be necessary for membrane ruffling in response to PAK1 activation (374), suggesting that RSK1 may play a similar role in actin reorganization.

Ran-binding protein 3 (RanBP3) phosphorylation by RSK modulates nucleocytoplasmic protein transport. Both RSK and Akt phosphorylate Ser58 of RanBP3, and that phosphorylation event contributes to the formation of a Ran gradient essential

for nucleocytoplasmic transport, kinetochore function, spindle assembly, microtubule dynamics, and other mitotic events (417). RSK phosphorylates the transcription/translation factor Y box-binding protein-1 (YB-1) at Ser102 and thereby promotes its nuclear accumulation (349). YB-1 is overexpressed in a number of cancer types, suggesting that RSK signaling may contribute to mediating the oncogenic functions of YB-1. RSK1 phosphorylates neuronal nitric oxide synthase (nNOS) on Ser847 in cells treated with mitogens, leading to the inhibition of NOS activity (342). Following EGF treatment, nNOS was phosphorylated by RSK1 in hippocampal and cerebellar neurons, suggesting a novel role for RSK in the regulation of nitric oxide function in the brain. RSK has been shown to phosphorylate the Akt substrate of 160 kDa (AS160), a protein implicated in the translocation of the glucose transporter 4 (GLUT4) to the plasma membrane in response to insulin (128). Deregulation of GLUT4 translocation occurs early in the pathophysiology of insulin resistance and type 2 diabetes.

MSK

Identification and protein structure. The mitogen- and stress-activated kinases 1 and 2 (MSK1 and MSK2) were discovered by two groups through genome-wide homology searches (83, 253). Simultaneously, MSK2 was detected in a yeast two-hybrid screen using p38α as bait (268). Human MSK1 (also known as RSK-like protein kinase or RLPK) and MSK2 (RSK-B) are 63% identical to each other and display significant homology to the RSKs (about 40% identity) (Fig. 5B). Similar to the RSKs, MSK1/2 possess two autonomous kinase domains within the same polypeptide (Fig. 6), a feature that is conserved in MSK orthologs from different species, such as *C. elegans* (C54G4) and *Drosophila* (JIL-1) (169). Although the role of C54G4 is unknown, JIL-1 is essential for viability in *Drosophila* and was found to mediate histone H3 phosphorylation (169). Similar to the RSKs, the NTKD of MSK1/2 belongs to the AGC family of kinases. The CTKD of MSK1/2 has a CAMK-like sequence and is mostly homologous to the kinase domain of MK2/3 (about 40% amino acid identity). Both MSK isoforms interact with ERK1/2 and p38 isoforms through their MAPK-binding domains (Leu-Ala-Lys-Arg-Arg-Lys), located near the C terminus of the protein (Fig. 3) (366).

Tissue expression and subcellular localization. Analysis of MSK1 and MSK2 expression in different tissues revealed that they are ubiquitously expressed, with predominant expression of MSK1 and MSK2 in the brain, heart, placenta, and skeletal muscle (83). Interestingly, the *Rps6ka4* gene (coding for MSK2) maps to the BBS1 locus on chromosome 11 (437). Bardet-Biedl syndrome (BBS) is a genetically heterogeneous disorder characterized primarily by retinal dystrophy, obesity, polydactyly, renal malformations, and learning disabilities (183). Although MSK2 inactivation has not yet been shown to contribute to BBS, it is worth noting that clinical symptoms of BBS are somewhat similar to those of CLS, a syndrome caused by RSK2 inactivation (Table 1).

The C terminus of MSK1/2 contains a functional bipartite NLS (Lys-Arg-Xaa$_{14}$-Lys-Arg-Arg-Lys-Gln-Lys in MSK2) (Fig. 4), conferring an almost exclusively nuclear localization in both serum-starved and stimulated cells (83, 268). Consistent with such localization, MSK1/2 have been shown to regulate mainly nuclear events (14, 378). Although MSKs do not appear to translocate following activation, expression of MSK2 was found to regulate the localization of ectopically expressed p38α and ERK1 (268). These results indicate that MSK1/2 may control the cellular localization of their upstream activators, ERK1/2 and p38, a finding that was also observed with the closely related MK2/3/5 (20).

Activation mechanisms and inhibitors. In cells, MSK1 and MSK2 are potently activated by mitogens and stress stimuli that promote ERK1/2 and p38 activation (83, 268). *In vitro*, both ERK1/2 and p38 directly phosphorylate the same sites on MSK1/2, resulting in their activation (83, 230). Consistent with these original observations, specific inhibitors of p38α and p38β (SB203580) and MEK1/2 (U0126, PD98059, and PD184352) block MSK1/2 activation in a stimulus-dependent manner (Fig. 7) (83, 366, 398). Contribution of the ERK5 module in MSK activation has not been studied in detail, but certain evidence suggests that ERK5 does not regulate MSK1/2 phosphorylation and activation (229).

MSK and RSK share many characteristics that first suggested they would have similar activation mechanisms (118). First, as stated above, they both contain two distinct and functional kinase domains, a rare characteristic for protein kinases (Fig. 4). Inactivation of either kinase domain through mutation of conserved residues completely blocks NTKD activity of MSK1/2 (83, 268, 367), indicating that unlike the case for the RSKs (29, 62, 302), MSK1/2 activation critically requires CTKD activation (62). Second, RSKs and MSKs share the four key phosphorylation sites essential for activation (Fig. 4), but regulation of these sites was found to be very different in MSKs. While PDK1-mediated phosphorylation of the NTKD was shown to be essential for RSK activation, analysis of *Pdk1*-null cells revealed that this kinase is not required for MSK1 activation (69, 399). Consistent with this, phosphorylation of the NTKD activation loop is mediated by autophosphorylation in MSK isoforms (229, 230). Whereas PDK1 is essential for activation of RSK1 to -3 (119), RSK4 appears to use autophosphorylation mechanisms similar to those of MSKs for activation (100), suggesting that they may share other similarities.

The current model of MSK activation suggests that MSK1/2 interact with ERK1/2 and p38 isoforms via the C-terminal MAPK docking site (Fig. 3). These upstream MAPKs then phosphorylate three Pro-directed residues in MSK1: a site in the linker between the two kinase domains (Ser360), the activation loop of the CTKD (Thr581), and a site in the C terminus (Thr700). Phosphorylation of Thr700 acts to reduce CTKD inhibition caused by an autoinhibitory C-terminal sequence (230). In combination with Thr581, phosphorylation of Thr700 activates the CTKD, which can then autophosphorylate two sites in the linker region (Ser376 and Ser381) as well as the activation loop of the NTKD (Ser212). This results in NTKD activation, which can then phosphorylate exogenous substrates (14, 378).

Based on their homology to the RSKs, the MSKs were predicted to contain additional phosphorylation sites in their C-terminal regions. Consistent with this, three residues (Ser750, Ser752, and Ser758) were found to be autophosphorylated by the NTKD upon MSK activation (229). While similar sites in RSK isoforms regulate ERK1/2 docking (302), these sites do not appear to play a similar function in MSK1 (229).

Although the MAPK-binding site of MSK1/2 is located near their C termini, MSK1 and MSK2 have an additional 20 to 50 aa C terminal to the docking site, suggesting that this region contains additional elements required for ERK1/2 and p38 association. Finally, several additional phosphorylation sites were recently identified in MSK1, but these are not required for MSK1 activation (230, 330). While the kinase(s) responsible for the phosphorylation of these sites remains to be identified, recent evidence suggest that casein kinase 2 (CK2) regulates MSK1 phosphorylation at multiple C-terminal residues following UV irradiation (162, 330).

Several compounds have been reported to inhibit MSK1/2 in cells (378), but none of them are selective, as they inhibit several kinases related to MSKs. MEK1/2 and p38 inhibitors can be used to prevent MSK activation, but both MAPK modules may need to be inhibited simultaneously to completely block MSK activation.

Substrates and biological functions. Although mice deficient in MSK1 and MSK2 do not display obvious phenotypes (15, 398), a relatively recent study indicated that MSK1/2-deficient animals are hypersensitive to lipopolysaccharide-induced endotoxic shock and showed prolonged inflammation in a model of toxic contact eczema (10) (Table 1). The authors of that study suggested that a lack of MSK1/2 signaling results in decreased expression of the phosphatase DUSP1 and the cytokine IL-10, which are normally involved in a negative feedback mechanism responsible for dampening the innate immune response.

The substrate specificity of the MSKs resembles that of other AGC family members, particularly RSK and PKA, with the minimal consensus substrate sequence Arg-Xaa-Xaa-pSer/Thr (83). In cells, both MSK and RSK can be activated by similar signals, suggesting possible redundancy in substrate phosphorylation. This may not be the case *in vivo*, however, as MSK1/2 localize primarily in the nuclei of quiescent and activated cells (83, 268), suggesting that they predominantly regulate nuclear substrates (Fig. 8). In addition, the MSKs do not require PDK1 in order to become activated, indicating that they may not depend on the same upstream pathways for activation. Studies from several groups indicated that MSK1/2 regulate gene expression at multiple levels, by playing active roles in transcriptional regulation and chromatin remodeling in response to stress and mitogens (14, 378). While deletion of both MSK isoforms in the mouse did not yield obvious developmental defects (398), this lack of a specific phenotype may be due to overlapping functions between AGC and MAPKAPK family members. This may not be the case in *Drosophila*, as deletion of the MSK ortholog JIL-1 is lethal (387), perhaps due to lower genetic redundancy (169). In *Drosophila*, JIL-1 is a key regulator of chromatin structure that functions to maintain euchromatic domains while counteracting heterochromatization and gene silencing (387). JIL-1 regulates histone H3 phosphorylation on Ser10, which was shown to inhibit the spread of heterochromatin markers to ectopic locations on the chromosome arms (19). These studies suggest that MSK1/2 play similar roles in gene regulation, which may affect several physiological functions, including both immunity and neuronal functions.

(i) Transcriptional regulation. MSKs phosphorylate many transcription factors and thereby increase their stability and/or activity (378). As mentioned for the RSKs, the transcription factor CREB was found to be an MSK1/2 substrate. Upon their discovery, MSK1/2 were shown to phosphorylate CREB on Ser133 *in vitro* with a K_m value much lower than those of PKA, RSK2, and MK2 (83, 268), raising the possibility that MSK1 rather than RSK2 mediates the mitogen-stimulated phosphorylation of CREB. Since then, work from several groups using different pathway inhibitors has supported the involvement of MSK1/2 in CREB phosphorylation (141, 207). The most convincing evidence for MSK-dependent phosphorylation of CREB comes from knockout mice. MEFs isolated from *Msk1*$^{-/-}$*Msk2*$^{-/-}$ mice were found to have greatly reduced CREB phosphorylation in response to stress and mitogens (398). The transcriptional activity of CREB requires its phosphorylation at Ser133, which recruits the CBP and p300 cofactors. Activated CREB participates in the transcriptional activation of several IE genes, such as those for c-Fos, JunB, and Egr1 (219). The knockout of both MSK1 and MSK2 resulted in a 50% reduction in c-Fos and JunB gene transcription in MEFs subjected to stress but only a minimal reduction in response to mitogenic stimulation (398), suggesting again that RSKs and MSKs may collaborate in the regulation of IE genes in response to mitogens. In addition to CREB, MSKs were also shown to regulate another CRE-binding factor, the activating factor 1 (ATF1). Phosphorylation of ATF1 at Ser63 appears to follow the same rule as CREB. Indeed, MSKs have been convincingly shown to be the primary ATF1 kinases in cells (398), but residual ATF1 phosphorylation in *Msk1*$^{-/-}$*Msk2*$^{-/-}$ cells suggests the involvement of a collaborating kinase.

The activity of the NF-κB transcription factor is highly regulated by posttranslational modification, and phosphorylation of the p65 subunit at Ser276 was attributed to MSKs (379). As with CREB, phosphorylation of p65 was first reported to be regulated by PKA, which associates with the IκB kinase complex (431). In this context, p65 phosphorylation was found to promote p65 interaction with the cofactors CBP and p300, which in turn acetylate histones and NF-κB bound to promoters (432). Loss of MSK1/2 in mouse fibroblasts resulted in reduced TNF-α-mediated activation of NF-κB (50, 379), suggesting that activation of MSK1/2 is important for NF-κB-dependent transcription.

Other transcription factors that may be regulated by MSKs include STAT3 (signal transducer and activator of transcription 3) and ER81. Phosphorylation of STAT3 at Ser727 in response to UV irradiation and erythropoietin (EPO) was shown to require MSK1 activity (397, 426). In the case of ER81, MSK1 was found to mediate its phosphorylation at Ser191 and Ser216, two residues that control its transcriptional activity (166). While STAT3 and ER81 may be *bona fide* substrates of MSKs, validation experiments with cells derived from *Msk1*$^{-/-}$*Msk2*$^{-/-}$ animals are still lacking. These will be necessary, as other MAPKAPKs also phosphorylate these transcription factors. MSK2 was also found to inhibit the transcription factor p53 in the absence of stress stimuli (218). This basal inhibition of p53 by MSK2 occurred independently of its kinase activity and of upstream MAPK signaling, suggesting that MSK2 acts to suppress p53 transcriptional activity under basal conditions.

(ii) Regulation of the chromatin environment. The primary roles of MSK1 and MSK2 may be as mediators of the nucleo-

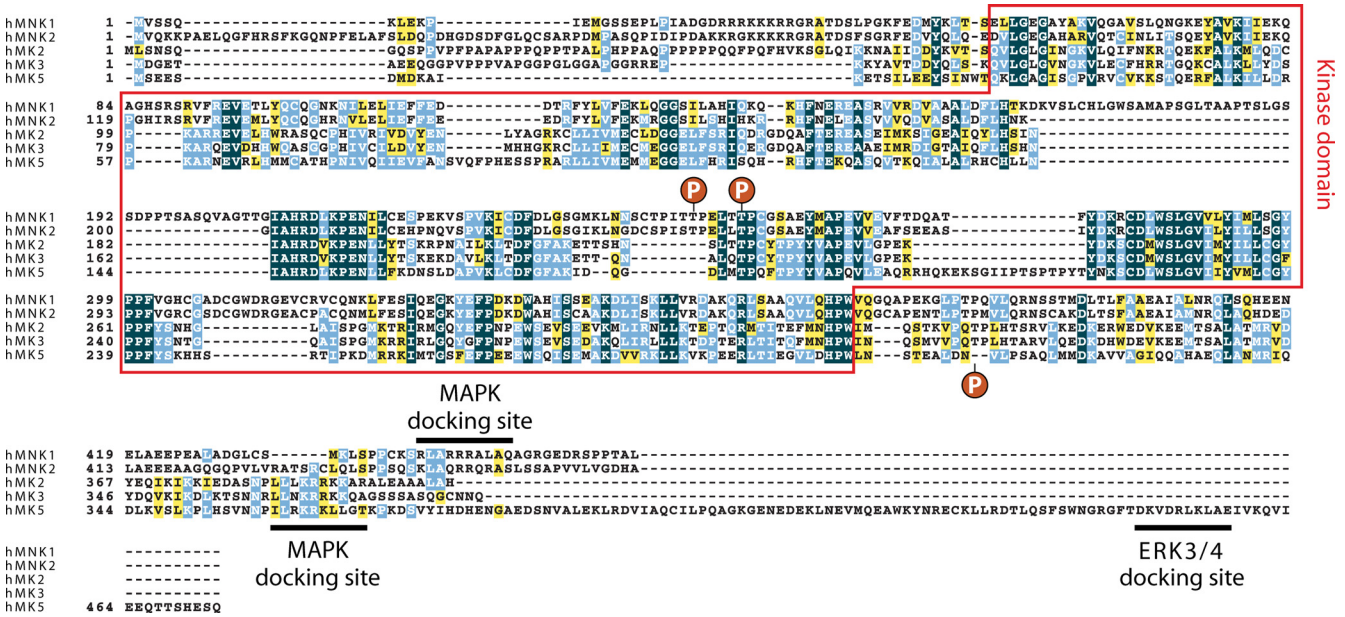

FIG. 9. Alignment of the amino acid sequence of the MAPKAPKs containing a single kinase domain. Sequences comprising the kinase domains and its subregions are boxed in red and reveal regions of highest homology. The conserved activation loop threonine residue is shown, as well as other conserved phosphorylation sites. The MAPK-binding domain is identified by a line.

somal response, which has the main goal of promoting gene relaxation and activation. The nucleosomal response refers to the rapid phosphorylation of histone H3 on Ser10 and of HMGN1 (also known as HMG-14) on Ser6 that occurs concomitantly with IE gene induction in response to a wide variety of stimuli (364). Histone H3 is a component of the nucleosome, and its C-terminal tail is the site of many posttranslational modifications, including phosphorylation, acetylation, methylation, and ubiquitination. Phosphorylation of histone H3 on Ser10 in response to mitogens and stress is associated with gene activation, suggesting that the ERK1/2 and p38 modules may regulate common nuclear effectors. Although RSK1 and RSK2 had initially been shown to phosphorylate histone H3 *in vitro* and *in vivo* (310, 315), the MSKs were later convincingly shown to be the primary histone H3 kinases (341). Indeed, stress- and mitogen-induced phosphorylation of histone H3 was found to be completely inhibited in primary embryonic fibroblasts from *Msk1*$^{-/-}$*Msk2*$^{-/-}$ animals (341), ruling out the possible involvement of other kinases such as the RSKs (81). Recently, MSK1-mediated phosphorylation of histone H3 at Ser10 was found to be required for tumor promoter-induced cell transformation (187).

In addition to Ser10, MSKs have also been shown to phosphorylate histone H3 on Ser28, a site also associated with IE gene induction (341). Although conserved from yeast to human, the molecular mechanisms by which phosphorylation of histone H3 regulates transcription remain unclear. Ser10 and Ser28 lie in putative 14-3-3-binding sequences, which may dislodge potential transcriptional repressors from activated promoter regions (223). Phosphorylation of histone H3 was found to inhibit binding of the transcriptional repressor HP1 (421), suggesting a possible mechanism by which MSK may regulate transcription (14). Alternatively, 14-3-3 proteins may act as scaffolds for the recruitment of SWI/SNF complexes at IE gene promoters and thereby promote chromatin remodeling upon activation of MSK1/2 (97).

As mentioned above, MSKs also regulate HMGN1, another chromatin-associated protein (341). While it is not a core component of nucleosomes, HMGN1 was shown to regulate chromatin compaction *in vitro* (155). Studies with HMGN1-null cells revealed that it suppresses histone H3 phosphorylation and IE gene expression (215), suggesting that MSK-mediated phosphorylation of HMGN1 may reduce its interaction with nucleosomes and thereby enable MSK1/2 to phosphorylate core histones (378).

(iii) Other substrates. Although this was not validated using cells derived from *Msk1*$^{-/-}$*Msk2*$^{-/-}$ animals, the MSKs have been suggested to phosphorylate the proapoptotic protein Bad (328) and the translational inhibitor 4E-BP1 (217).

MNK

Identification and protein structure. The MAPK-interacting kinases 1 and 2 (MNK1 and MNK2) were discovered simultaneously by two teams in 1997. The first group used a two-hybrid screen designed to identify ERK2-binding proteins (389). The second approach consisted of a phosphorylation screen looking for ERK1 substrates (121). The catalytic domain of human MNK1 displays approximately 70% amino acid identity with MNK2 (Fig. 9), and MNK orthologs have been identified in other species. MNK1 displays relatively high homology (51% amino acid identity) to a large protein kinase in *Drosophila* termed LK6, which was found to localize to centrosomes and to regulate microtubule organization (185). MNK1 also displays sequence homology (46% amino acid identity) with the *C. elegans* protein kinase mnk-1 (R166.5),

and knockdown of this protein is embryonic lethal in the worm (343). The catalytic domain of MNK1/2 belongs to the CAMK family of kinases and is most similar to the CTKD of the RSKs (33% amino acid identity) and MK2/3 (389) (Fig. 4). Compared to those of other MAPKAPKs, the kinase domains of MNK1 and MNK2 have several particularities. First, the MNKs have a DFD (Asp-Phe-Asp) motif in subdomain VII, whereas most protein kinases, including MAPKAPK family members, have DFG (Fig. 5A). Second, the kinase domain of MNK1/2 has two unusual short inserts, one in the activation loop (Fig. 5A) and the other after the APE motif of subdomain VIII (44). Interestingly, these features are also present in *Drosophila* LK6, suggesting that they may serve a function in MNK activation and/or substrate recognition.

Tissue expression and subcellular localization. Both the *Mnk1* and *Mnk2* genes generate two spliced isoforms, a long form (MNK1A and MNK2A) and a shorter version (MNK1B and MNK2B) that lacks the C-terminal MAPK-binding motif (259, 335). The MAPK-binding domain of MNK1A consists of Leu-Ala-Arg-Arg-Arg (Fig. 3) and mediates the interaction of MNK1 with both ERK1/2 and p38 (121, 389). The D domain present in MNK2A contains only two contiguous basic residues (Leu-Ala-Gln-Arg-Arg) and is very similar to the motif found in RSKs (Fig. 3). Consistent with this, MNK2A preferentially interacts with ERK1/2 (389).

Both MNK isoforms contain a polybasic sequence lying N terminal to the kinase domain (Fig. 4) (312). This sequence is involved in the recognition of eIF4G, as is discussed below, but also serves as an NLS that promotes MNK nuclear import (263, 275). Indeed, using a two-hybrid strategy, the NLS of MNK1 has been shown to mediate binding to importin α, which is an intracellular receptor protein involved in nuclear import (263, 390). Although all MNKs contain this sequence, not all isoforms are nuclear, and this is partly due to the presence of a CRM1-type NES in their C termini (263). The NES of MNK1 was shown to be functional through the use of leptomycin B (which blocks CRM1-dependent nuclear export), as treatment with the drug traps MNK1 in the nucleus and revealed that MNK1 is actively shuttling between the cytoplasm and the nucleus (231, 263). The C termini of MNK1B and MNK2B do not contain such a motif, which correlates with their mostly nuclear localization (259, 312). Because MNK2A lacks critical residues important for NES function (Fig. 4), its cytoplasmic localization is more enigmatic. Data suggest that its extreme C-terminal domain interferes with binding of its polybasic region to importin α, which may affect its entry into the nucleus (312).

The expression of MNK1 and MNK2 has not been studied extensively, but both proteins are expressed in all adult tissues, with the exception of the brain, where levels are greatly reduced compared to those in other tissues (389). The expression of both proteins was shown to be especially abundant in skeletal muscle, suggesting a higher requirement for MNK activity in this tissue.

Activation mechanisms and inhibitors. The basal activities and regulation of MNKs by MAPK agonists vary depending on the isoform. MNK1A has low basal activity in cells and is responsive to agonists of both ERK1/2 and p38 isoforms (Fig. 7) (121, 384, 389, 390). In contrast, MNK1B has high basal activity in quiescent cells, and this activity is not significantly affected by inhibitors of the ERK1/2 and p38 modules (258), consistent with the fact that MNK1B does not contain a MAPK-binding domain. With respect to MNK2, the long isoform (MNK2A) displays high basal activity in quiescent cells, and this activity can be slightly enhanced by agonists of ERK1/2 but not p38 (311). MNK2B has very low activity under most types of stimulation, and it is unclear which circumstances will promote its activity (312). There are several potential reasons for differences in the levels of activity between MNK1A and MNK2A, some of which involves features of both the C-terminal region and the catalytic domain. This was nicely illustrated through the generation of MNK1/2 chimeras with inverted C-terminal regions (262).

Phosphopeptide analysis of MNK1 and MNK2 revealed the presence of several MAPK-stimulated phosphorylation sites (311, 390). Phosphorylation of two proline-directed sites within the activation loop of MNKs (Thr209 and Thr214 in MNK1A) was found to be essential for activation (Fig. 4), as replacement of both sites by Ala residues in MNK1/2 results in inactive kinases (311). Another important residue was found to be phosphorylated in MNK1/2 (Thr344 in MNK1A), but mutation of this phosphorylation site yielded different outcomes in MNK1 and MNK2. While replacement by an Ala residue did not affect MNK1A but completely disrupted MNK2A activity, replacement of Thr344 by an Asp residue resulted in a constitutively activated MNK1A enzyme while not affecting MNK2A activity (121, 311, 389). Deletion of the C-terminal 91 aa containing the MAPK-binding motif was also shown to render MNK1A inactive (121), suggesting that ERK/p38 docking and phosphorylation of several important regulatory sites are required for efficient MNK1 and MNK2 activation.

The compound CGP57380 has been described as an MNK inhibitor (190) and used in cell-based assays for this purpose in several studies. However, a recent study indicated that this compound was a relatively weak inhibitor of MNKs, with 50% inhibitory concentrations (IC_{50}s) in the low-micromolar range (18). In addition, CGP57380 was tested against an extended panel, and many protein kinases were inhibited with similar potencies, including MKK1, CK1, and BRSK2. These studies indicated that CGP57380 is not a specific inhibitor of MNK isoforms, and results obtained from its use in cell-based assays should be interpreted with caution. The use of cells derived from *Mnk1*$^{-/-}$*Mnk2*$^{-/-}$ knockout animals (373) should provide valuable information and should be favored over the use of CGP57380 to validate substrates and biological functions.

Substrates and biological functions. MNKs have been reported to phosphorylate a number of different substrates, including components of the translational machinery and some mRNA-binding proteins and splicing factors (Fig. 8). The exact consensus phosphorylation motif of MNKs has not been determined, and even based on known phosphorylated sites in MNK substrates, no clear consensus that would help for prediction of sites emerges.

(i) eIF4E and eIF4G. Regulation of protein synthesis by the eukaryotic initiation factor 4F (eIF4F) complex plays an important role in controlling cell growth and proliferation (reviewed in reference 286). In higher eukaryotes, the eIF4F complex consists of three subunits: eIF4A, eIF4E, and eIF4G. eIF4E recruits mRNAs to the complex through binding of their 5′-cap structure, the N^7-methylguanosine cap. eIF4E also

interacts with the scaffold protein eIF4G, which recruits the RNA helicase eIF4A to the complex, unwinds the secondary structure in the mRNA, and facilitates ribosome scanning to the initiating codon. eIF4E is phosphorylated on Ser209 in response to stress and mitogen stimulation, and this phosphorylation event was shown to be dependent on both the ERK1/2 and p38 modules (115). MNK1 was found to be recruited to the eIF4F complex through its association with the C terminus of eIF4G (276), making it a likely candidate kinase for eIF4E. Consistent with this idea, MNK1 and MNK2 were shown to phosphorylate eIF4E at Ser209 in response to stress and mitogen stimulation (190, 311, 389). More recently, this phosphorylation event was shown to be completely absent in cells derived from *Mnk1*$^{-/-}$*Mnk2*$^{-/-}$ animals, indicating that the MNKs are the predominant eIF4E kinases operating in cells (373). eIF4G was also suggested to be phosphorylated by MNK1/2 (275), but more experimentation will be required to confirm this regulation and its biological significance.

Although eIF4E phosphorylation has been studied for over 2 decades, no clear consensus has emerged regarding its biological significance, and this topic has been highly debated (313). Phosphorylation of eIF4E has been shown to decrease its affinity for the cap structure of mRNA (314), but the exact role of eIF4E phosphorylation in the regulation of translation remains elusive. Several lines of evidence suggest that MNKs are negative regulators of protein synthesis. Overexpression of MNK was found to decrease cap-dependent translation in *Aplysia* neurons (294), suggesting that hyperphosphorylation of eIF4E decreases protein synthesis rates. Treatment of cells with the MNK1/2 inhibitor CGP57380 was found to slightly enhance cap-dependent translation (190), consistent with the idea that MNKs may play a negative role in eIF4E-dependent translation. Conversely, recent work has suggested that MNK1/2 positively regulate mRNA translation and polysome assembly (27, 425), but these studies generally relied on CGP57380 to inhibit MNK1/2 activity and eIF4E phosphorylation, and as indicated above, this inhibitor is not very selective and may have pleiotropic effects. Despite having undetectable eIF4E phosphorylation at Ser209, mice deficient in MNK1 and MNK2 develop normally, and cells derived from these animals have normal global protein synthesis and cap-dependent translation (373). These findings suggest that the role of eIF4E phosphorylation may not be evident under normal growth conditions and that MNK1/2-dependent eIF4E phosphorylation may be involved in more specialized functions, such as in response to oncogene activation and cellular stress. Consistent with this idea, expression of a constitutively activated form of MNK1 was shown to promote tumorigenesis in the mouse, through a mechanism that may involve expression of the antiapoptotic protein Mcl-1 (392).

(ii) Other substrates. Two RNA-binding proteins were shown to be regulated by MNKs, the heterogeneous nuclear ribonucleoprotein A1 (hnRNP A1) and the polypyrimidine tract-binding protein (PTB)-associated splicing factor (PSF). hnRNP A1 is a very abundant nuclear protein that plays an important role in mRNA metabolism. The MNKs were shown to phosphorylate hnRNP A1 at two potential sites, Ser192 and Ser310, in response to T-cell activation (45). Phosphorylation of hnRNP A1 was found to decrease its ability to interact with the TNF-α mRNA, suggesting that MNKs may play key roles in the regulation of specific messages. hnRNP A1 also becomes phosphorylated in response to osmotic stress in a p38-dependent manner (377), suggesting that MNKs may also regulate translation of specific mRNAs under stress conditions. PSF is a nuclear protein involved in transcription and RNA processing. Together with p54^{nrb}, PSF forms a transcription-splicing factor implicated in diverse biological functions. MNKs were shown to phosphorylate PSF at two sites *in vitro*, Ser8 and Ser283, which increase its affinity *in vivo* for the TNF-α mRNA (43), suggesting again that MNKs may regulate the fate of specific mRNAs.

Cytoplasmic phospholipase A_2 (cPLA$_2$) plays a key role in the production of eicosanoids, which are second messengers with important functions in immunity and inflammation. MNK1 was shown to phosphorylate cPLA$_2$ on Ser727 *in vitro* (150), a phosphorylation event that depends on the p38 module in cells. Phosphorylation of cPLA$_2$ on Ser727 was shown to correlate with enzyme activation, suggesting that MNK1 may regulate cPLA$_2$-mediated arachidonate release. It is important to note that the p38-activated protein kinases MSK1 and MK5 were also shown to phosphorylate cPLA$_2$ *in vitro*, suggesting that other MAPKAPK family members may participate in the phosphorylation of Ser727 and regulation of cPLA$_2$. Finally, MNK1 has been shown to regulate the phosphorylation of Sprouty 2 (Spry2), a membrane-associated protein that suppresses ERK activation and/or signaling (42). Recent work shows that MNK1 phosphorylates Spry2 on two sites, Ser112 and Ser121, and replacement of these sites by Ala residues caused destabilization of Spry2 (79). The MNK inhibitor also led to degradation of Spry2, suggesting that MNK1-mediated phosphorylation of Spry2 may prolong its half-life and thus its capacity to inhibit ERK signaling.

MK2/3

Identification and protein structure. The MAPK-activated protein kinase 2 (MK2) was discovered as an ERK1/2-activated protein kinase that could phosphorylate heat shock protein 25 (Hsp25) and Hsp27 (346). It was 2 years later that two groups determined that MK2 was in fact stimulated by the p38 module in response to stress stimuli (117, 295). MAPK-activated protein kinase 3 (MK3) was discovered a few years later by two groups, using a two-hybrid screen for p38-interacting proteins (232) and by analyzing genes commonly deleted in small-cell lung cancer (334). A single homolog kinase of MK2/3 has been identified in *Drosophila* and *C. elegans*, which share about 60% amino acid identity with human MK2 (204). Yeasts lack obvious structural homologues of MK2, but functionally homologous kinases that also respond to p38 (Hog1 in yeast) do exist; these are Rck1 and Rck2 in budding yeast (279) and Srk1 (also known as Mkp1) and Mkp2 in fission yeast (16).

MK2 is highly homologous to MK3 (75% amino acid identity), and their kinase domains are most similar (35 to 40% identity) to CAMK, and the CTKD of RSK (347, 438) (Fig. 9). Vertebrate MK2/3 contain an N-terminal proline-rich region that interacts with the Src homology 3 (SH3) of c-Abl *in vitro* (270). The C terminus of MK2 contains a functional bipartite NLS (Lys-Lys-Xaa$_{10}$-Lys-Arg-Arg-Lys-Lys) that is also present in MK3 (Fig. 3). The NLS found in both MK2 and -3 encompasses a D domain (Leu-Leu-Lys-Arg-Arg-Lys-Lys in MK2)

(Fig. 4) that mediates specific interaction with p38α and p38β (337). MK2 and MK3 also possess a functional NES (Met-Thr-Ser-Ala-Leu-Ala-Thr-Met-Arg-Val) located N terminal to the NLS and D domain (Fig. 4). The NES in MK2 triggers its nuclear export following stimulation, which can be inhibited by leptomycin B (20, 104).

Tissue expression and subcellular localization. MK2 and MK3 mRNAs are expressed at detectable levels in most tissues analyzed, with a predominant expression in the heart, skeletal muscle, and kidney (105, 334, 346). The *Mk2* gene was shown to give rise to two alternatively spliced transcripts (58, 346), and more recently, a similar observation was made with the mouse *Mk3* gene (240). The shorter MK2 and MK3 isoforms (MK2S and MK3S) lack part of the C-terminal region and thus the nuclear import/export sequences and the MAPK-binding domain. MK3S also lacks catalytic subdomains IX, X, and XI but, surprisingly, displayed detectable *in vitro* kinase activity toward Hsp27 (240). MK2S is not well represented in expressed sequence tag (EST) databases, suggesting that this isoform may be the result of a rare alternative splicing event (122).

While MK2S and MK3S are mostly cytoplasmic enzymes (240, 438), MK2 and MK3 are located predominantly in the nuclei of quiescent cells (104, 250). Upon stress stimulation, both enzymes are rapidly exported to the cytoplasm using a CRM1-dependent mechanism (20, 104, 321). While the long form of MK3 translocates upon stimulation, MK3S is degraded in a stress- and p38-dependent manner (240). Intramolecular unmasking of the NES found in MK2/3 was shown to be regulated by phosphorylation. Indeed, nuclear export of MK2 appears to be mediated by kinase activation, as phosphomimetic mutation of Thr334 enhances the cytoplasmic localization of MK2 (104). For this reason, it is thought that MK2 and MK3 contain a constitutively active NLS and a phosphorylation-regulated NES.

Activation mechanisms and inhibitors. MK2 and MK3 are activated under various stress conditions that stimulate the p38 isoforms (117, 139, 232, 295), such as UV irradiation, heat shock, oxidative stress, hyperosmolarity, and different cytokines. Through the use of a p38-specific inhibitor (SB203580) (66) and p38α-deficient cells (6), MK2 activation was found to be completely dependent on p38α (Fig. 10). Activated p38α promotes MK2 phosphorylation at Thr222 within its activation loop (Fig. 5A), Ser272 within subdomain X of the kinase domain, and Thr334 located in a hinge region that controls MK2 autoinhibition (21). The crystal structure of MK2 has been resolved (394) and suggests that Thr334 phosphorylation may serve as a switch for MK2 nuclear import and export (236). Upon activation, phosphorylation of Thr334 is thought to release the autoinhibitory helix from the core of the kinase domain, thereby exposing the NES and promoting nuclear export (104). This MK2 conformational change was demonstrated by fluorescence resonance energy transfer (FRET) analysis of a green fluorescent protein (GFP)-MK2 fusion protein, which revealed that MK2 activation correlates with an open conformation that is detectable only in the cytoplasm of activated cells (249). In contrast to the regulated NES, the C-terminal NLS is active independently of MK2 phosphorylation state, allowing this kinase to shuttle between the nucleus and the cytoplasm. As part of the NLS overlaps with the MAPK docking domain, it is possible that p38 docking regulates NLS function, but this has not been addressed.

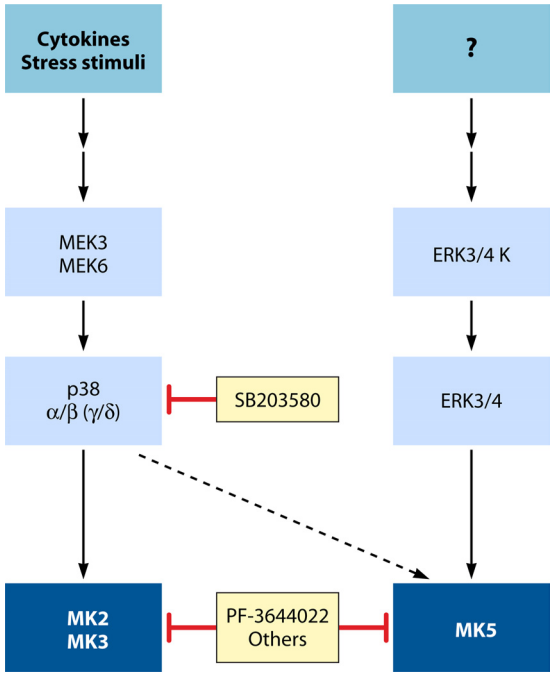

FIG. 10. Signaling cascades leading to activation of MK2/3 and MK5. MK2/3 have been shown to be activated by both ERK1/2 and p38 kinases. Conversely, MK5 was initially shown to be regulated by p38, but recent data suggest a stronger link with ERK3 and ERK4. Different inhibitors of components within these cascades are also shown. Dotted lines indicate that, although reported, substrate regulation by the respective kinase remains to be thoroughly demonstrated.

MK2 forms a stable complex with p38α, and each protein mutually stabilizes its partner (122). Indeed, knockout studies revealed decreased levels of p38α in MK2-deficient cells (196), and MK2 expression was shown to be reduced in p38α-deficient cells (350). The reason for this is unclear, but it was suggested that MK2 may compete with other partners of p38α involved in its inactivation. Early studies have suggested that p38α is exported from the nucleus in a complex with MK2 (20), but more recent findings indicate that this is unlikely (293). A large number of studies have used p38 inhibitors to prevent MK2/3 activation and study their biological functions. However, several pharmaceutical companies have recently reported new small-molecule inhibitors of MK2 (124), some of which are reversible ATP-competitive compounds (e.g., PF-3644022) that suppress MK2-dependent functions in cells (243).

Substrates and biological functions. Experimental evidence supports a role for the p38 module in cytokine production (142, 208), cell migration (148, 269), actin remodeling (296), cell cycle control (9), and gene expression (87). Interestingly, targeted deletion of the mouse *Mk2* gene provided convincing evidence that although p38 regulates a large set of substrates, MK2 appears to be a key player in these p38-dependent biological processes (122, 293).

MK2 and MK3 have comparable substrate preferences and phosphorylate the same residues in Hsp25 and Hsp27 with

similar kinetic constants (66). The optimal sequence required for efficient phosphorylation by MK2/3 is Φ-Xaa-Arg-Xaa-(Leu/Asn)-pSer/Thr-(Iso/Val/Phe/Leu)-Xaa, where Φ is a bulky hydrophobic residue (66, 227, 334). While MK2 and MK3 appear to be equals *in vitro*, the *in vivo* situation is quite different, as MK2 activity was shown to be higher than that of MK3 (292). Consistent with this, MK3 was shown to partly compensate for the loss of MK2 in mice, whereas deletion of MK3 alone had very little effect (Table 1). MK2 and MK3 regulate a number of substrates, some of which are described in detail below (Fig. 8).

(i) Actin remodeling and cell migration. The first MK2/3 substrates to be identified were Hsp25 and Hsp27 (221, 232, 348), which are ATP-independent chaperones that keep unfolded proteins in a folding-competent state before they can be refolded by Hsp70 (194). Hsp27 is of particular interest because it forms large oligomers which can act as molecular chaperones and protect cells from heat shock and oxidative stress (203). Once phosphorylated by MK2/3 at several residues, Hsp27 loses its ability to form large oligomers and is unable to block actin polymerization (203, 290). These findings suggest that MK2 serves a homeostatic function aimed at regulating actin dynamics that would otherwise be destabilized during stress (113, 139). Other MK2/3 substrates that are involved in actin remodeling include the p16 subunit (p16-Arc) (333) of the seven-member actin-related protein 2/3 complex (Arp2/3), lymphocyte-specific protein 1 (LSP-1) (157), and F-actin-capping protein Z-interacting protein (CapZIP) (110). Others, such as vimentin (57) and αβ-crystallin (182), may interact with intermediate filaments and microtubules. Remodeling of the actin cytoskeleton is a prerequisite for cell migration, and phosphorylation of Hsp27 by MK2/3 was shown to contribute to cell motility (237). Consistent with this, MK2-deficient neutrophils have defects in chemotaxis and altered chemokinesis (146). The exact contribution of other MK2/3 substrates involved in actin remodeling remains elusive.

(ii) Cytokine production. Following stimulation of the p38 module with LPS, MK2 regulates the inflammatory response through posttranscriptional mechanisms. MK2 was found to increase production of TNF-α and IL-6 by promoting translation and/or stability of their mRNAs (195, 248). MK2-deficient mice are less sensitive to LPS-induced endotoxic shock but more susceptible to bacterial infection (211), consistent with an impaired inflammatory response. While deletion of MK3 alone does not significantly alter cytokine production, absence of both MK2 and MK3 further impairs the inflammatory response, suggesting significant overlap in function (292). The stability of many mRNAs, including those of IL-6 and TNF-α, depends on AU-rich elements (AREs) located in their 3′ untranslated regions (3′-UTRs). Several proteins bind ARE-containing mRNAs, many of which are specifically regulated by MK2/3 (248, 401). Consistent with this, MK2 has been shown to bind and/or phosphorylate hnRNP A0 (297), tristetraprolin (TTP) (225), poly(A)-binding protein 1 (PABP1) (33), human R-antigen (HuR) (144, 369), and butyrate response factor 1 (BRF1) (226). MK2-dependent phosphorylation of TTP creates functional 14-3-3-binding sites (61) that inhibit TTP-dependent degradation of ARE-containing transcripts and thereby contributes to LPS-induced TNF-α expression (38, 154, 345). Alternatively, MK2-mediated phosphorylation of TTP may reduce its ability to promote deadenylation by inhibiting the recruitment of the CAF1 deadenylase, as suggested in a recent study (228).

(iii) Transcriptional regulation. The polycomb group family, originally identified in *Drosophila* as a repressor of homeotic genes, represents epigenetic chromatin modifiers with a transcriptional silencing function (376). Recent evidence indicates that polycomb group proteins may be targets for MK2 (414) and MK3 (381). Indeed, MK2/3 bind the human polyhomeotic protein 2 (HPH2), which is a component of the large and dynamic polycomb repressive complex 1 (PRC1). Following stress stimulation, MK2/3 phosphorylate components of the complex, such as BMI1, a protein involved in the self-renewal of hematopoietic stem cells. Interestingly, a recent study demonstrated that hematopoietic stem cells in MK2-deficient mice are reduced in number and show an impaired ability for competitive repopulation *in vivo* (320).

In addition, MK2 was shown to contribute to the phosphorylation of SRF (151) and ER81 (165). Comparison of wild-type and MK2-deficient cells revealed that MK2 is the major SRF kinase induced by stress (151), suggesting a role for MK2 in stress-mediated IE gene response. Both MK2 and -3 interact with the basic helix-loop-helix (bHLH) transcription factor E47 *in vivo* and phosphorylate E47 *in vitro* (250). MK2-mediated phosphorylation of E47 was found to repress E47 transcriptional activity, suggesting that MK2/3 may regulate tissue-specific gene expression and cell differentiation (250).

(iv) Cell cycle control. MK2 has recently been shown to phosphorylate CDC25B and CDC25C in response to UV irradiation (227). CDC25C is an evolutionarily conserved dual-specificity phosphatase that directs dephosphorylation of cyclin B-bound Cdc2 and triggers entry into mitosis (13). Upon DNA damage, CDC25 is inhibited by the Chk1 and Chk2 protein kinases, and MK2 appears to work in parallel to these enzymes to promote the G_2/M checkpoint in response to stress (227). Intriguingly, RSK was also found to phosphorylate CDC25C in *Xenopus* oocytes (63), suggesting that RSK may also contribute to a G_2/M cell cycle arrest under certain circumstances.

p53 stability is regulated by HDM2, a RING domain protein that acts as an E3 ligase to ubiquitinate p53 and target it for degradation. Phosphorylation of HDM2 on Ser166 by Akt was shown to enhance HDM2 activity and promote the degradation of p53 (433). Interestingly, MK2 was also found to phosphorylate HDM2 on Ser157 and Ser166, resulting in HDM2 activation and degradation of p53 in response to stimuli of the p38 module (391). These results suggest that MK2 may act to dampen the extent and duration of the p53 response to stress and DNA damage, but more experimentation will be required to test this model. Consistent with this scenario, a recent study showed decreased HDM2 phosphorylation and increased p53 levels in MK2-deficient mice subjected to a skin carcinogenesis model (171).

(v) Other substrates. 14-3-3 proteins normally interact with a number of signaling pathway components, including protein kinases, phosphatases, and transcription factors (reviewed in reference 242). The generation of 14-3-3-binding sites appears to be important for MK2-dependent functions, as 14-3-3 interacts with several MK2 substrates in a stress-inducible manner. In addition to TTP, which was described above, MK2 promotes 14-3-3 binding to Hsp27 (96), CDC25B and CDC25C (227),

and TSC2 (214) to modulate their function. TSC2 normally functions as a negative regulator of mTOR activation, and MK2-mediated phosphorylation of TSC2 at Ser1210 may regulate mTOR-dependent protein synthesis in response to certain stresses.

MK5

Identification and protein structure. MK5 (also known as p38-regulated/activated protein kinase [PRAK]) was discovered simultaneously by two groups searching EST databases for proteins with sequence homology to MK2 (252, 254). While MK2 and MK3 display 75% amino acid identity, MK5 is more distantly related to MK2/3, with 38% homology to these enzymes (Fig. 9). MK5 and MK2/3 may have originated from a common ancestral protein, because all three proteins display similar amino acid identities to *Drosophila* and *C. elegans* homologs of MK2. Like that of MK2/3, the kinase domain of MK5 is most similar to CAMK-related kinases (Fig. 4).

MK5 has a structure similar to that of MK2/3, consisting of a kinase domain flanked by a short N-terminal region lacking the SH3 domain-binding motif and a 100-aa C-terminal extension that contains a functional NLS, NES, and MAPK-binding motif (Fig. 4). The NLS (Arg-Lys-Arg-Lys) of MK5 is functional, as Ala substitutions disrupt its nuclear localization (251, 321). The NLS also overlaps with a MAPK docking site (Ile-Leu-Arg-Lys-Arg-Lys-Leu-Leu) that mediates interaction with p38α and p38β (Fig. 3) (321). Interaction of p38 with MK5 was found to be weaker than that with MK2, and consistent with this, MK5 does not appear to stabilize p38 *in vivo* (331). The NES of MK5 (Leu-Arg-Val-Ser-Leu-Arg-Pro-Leu-His-Ser) is also functional and is sufficient to trigger CRM1-dependent nuclear export (321). Finally, the C-terminal region of MK5 contains a new domain necessary for interaction with ERK3 and ERK4 (5).

Tissue expression and subcellular localization. MK5 expression appears to be ubiquitous, with predominant expression in the heart, skeletal muscle, pancreas, and lung (130, 252, 254, 267). The human *Mk5* gene encodes two alternatively spliced transcripts of 471 and 473 aa, but the relevance of having two nearly identical isoforms is currently unknown. In mice, four MK5 isoforms have been identified, in addition to the full-length protein, resulting from both exon skipping and alternative splice site activation during pre-mRNA processing (91). The biological significance of these different isoforms is still unknown, but these proteins appear to have distinct subcellular localizations, catalytic activities, and tissue expression. When overexpressed, full-length MK5 localizes to the nuclei of quiescent cells, but upon cellular stress, MK5 translocates to the cytoplasm (251, 321). Interestingly, expression of exogenous p38α with MK5 has been shown to relocalize MK5 to the cytoplasm, suggesting that p38 docking interferes with the function of its NLS (321).

Activation mechanisms and inhibitors. Despite MK5 and MK2/3 being structurally related, the activation mechanisms of MK5 are quite different from those of MK2/3 (Fig. 10). Several groups have shown that under conditions of overexpression, p38 phosphorylates MK5 in response to cellular stress (251, 252, 254, 321). Analysis of *in vitro*-phosphorylated MK5 revealed that p38 phosphorylates the activation loop residue of MK5 (Thr182) (Fig. 5A), which is essential for kinase activation (252). However, certain experimental evidence suggests that MK5 is not a physiological p38 substrate. First, endogenous MK5 is not significantly activated by classical p38 stimuli, such as arsenite and sorbitol (331). Second, MK5 is not involved in the inflammatory response induced by LPS, a strong agonist of the p38 module (331). Indeed, MK5-deficient mice are not resistant to endotoxic shock caused by LPS and do not have impaired cytokine production. Third, no interaction between endogenous MK5 and p38 has ever been reported (322, 331, 351).

More recently, MK5 was also shown to be a substrate of ERK3 and ERK4. These atypical MAPKs regulate MK5 activity when overexpressed, but unlike for the p38 isoforms, this was also demonstrated under physiological conditions (4, 179, 318, 322). Indeed, endogenous MK5 kinase activity was shown to be reduced in *Erk3*$^{-/-}$ MEFs (322), and the interaction between ERK3/4 and MK5 was demonstrated at the endogenous level (179, 322). While ERK3/4 phosphorylate MK5 at its activation loop residue Thr182 (Fig. 5A), ERK3 was also shown to act as a scaffolding protein by promoting the autophosphorylation and activation of MK5 (318). ERK3 and ERK4 can also regulate the subcellular localization of MK5 (4, 179, 318, 322). When expressed independently, MK5 and ERK3 localize mainly in the nucleus, whereas ERK4 displays a cytoplasmic localization. Coexpression of MK5 with ERK3 or ERK4 promotes MK5 relocalization to the cytoplasm, and this event was shown to be facilitated by activation loop phosphorylation of ERK3 and ERK4 (86, 266).

Substrates and biological functions. The exact biological function of MK5 is unknown, but recent evidence suggests that MK5 may play a role in oncogene-induced senescence (351) and actin remodeling (129) (Fig. 8). MK5 was originally thought to share many substrates with MK2, such as Hsp27 and glycogen synthase (252), but this was questioned by the characterization of the MK5-deficient mouse (331). Despite the relatively high similarity between MK2/3 and MK5 (Fig. 9), MK5-deficient mice do not display any of the phenotypic changes seen in MK2-deficient animals (195). Indeed, disruption of the *Mk5* gene in mice of mixed genetic background did not give rise to detectable phenotypic changes (331) (Table 1). However, inactivation of MK5 into a C57BL/6 genetic background resulted in lethality at embryonic day 11.5 (E11.5) with incomplete penetrance (318). ERK3 and MK5 mRNAs were shown to be coexpressed in space and time during mouse embryogenesis, suggesting an important role for this signaling module during development.

(i) Tumor suppression. Certain observations suggest that ERK3 may play some roles in tumor suppression, including its apparent negative regulatory effect on cell cycle progression, cell proliferation, and migration (73, 75, 173). MK5 may be an important ERK3 effector protein mediating these effects, as it was recently shown to promote tumor suppression and oncogene-induced senescence (351). Indeed, MK5-deficient mice from a mixed genetic background were found to be more susceptible to skin carcinogenesis induced by the mutagen dimethylbenzanthracene (DMBA), an agent that causes skin tumors that are 90% positive for Ras mutations. Consistent with these findings, MK5 was shown to be essential for Ras-induced senescence in primary mouse and human fibroblasts. Reintro-

duction of a kinase-defective MK5 mutant could not restore Ras-induced senescence in MK5-deficient cells, suggesting that MK5 may regulate an essential substrate involved in cellular senescence. The same group demonstrated that MK5 phosphorylates p53 at Ser37, a residue that promotes its transcriptional activity (351). Interestingly, this residue is not located within a consensus for MAPKAPKs but rather is followed by a Pro residue, suggesting that this site is a more likely candidate for phosphorylation by MAPKs. Although the role of MK5 in oncogene-induced senescence appeared to require activation of the p38 module, the potential roles of ERK3 and ERK4 were not determined in that study, and there may be important roles for these atypical MAPKs.

(ii) Actin remodeling. A potential role for MK5 in cytoskeletal organization was also suggested. Overexpression of MK5 was reported to increase HeLa cell migration and F-actin production (354). The same study also showed that 14-3-3ε interacts with and inhibits MK5, resulting in reduced phosphorylation of Hsp27, cell migration, and actin filament dynamics. Similar findings were observed in PC12 cells, where knockdown of MK5 reduced forskolin-induced F-actin levels (129). Recent evidence suggests that F-actin rearrangement requires MK5-mediated phosphorylation of Hsp27 (193, 194), but more experimentation with genetic models lacking MK2/3 or MK5 will be required to confirm the role of MK5 in actin remodeling.

CONCLUSIONS AND PERSPECTIVES

MAPKAPK family members display relatively high homology and are activated by similar mechanisms involving conserved sequences in their kinase domains. Despite these facts, activation of each MAPKAPK family member results in regulation of specific substrates and cellular functions. While all MAPKAPKs appear to have independent functions, these kinases may also act in concert to mediate global biological responses. For example, MAPKAPK activity may generally be needed under mitogenic or stressful conditions, where, for example, stress-induced p38 activation results in increased cytokine production. In this instance, cytokine gene transcription may require chromatin remodeling by MSKs, increased mRNA stability by MK2/3, and increased translation mediated by MNKs.

Many reports have established the role of MAPKAPKs in various biological processes, including the response to mitogens, oncogenes, stress, and inflammation, as well as the regulation of proliferation, differentiation, and survival in particular cell types. There is also ample information on pathway components and their regulatory mechanisms. However, very little is known about *in vivo* functions, and detailed molecular information on how these signaling molecules regulate particular cellular processes is still scarce. Outstanding questions that should be addressed in the future include (i) the extent of functional redundancy and interplay between MAPKAPK family members, (ii) how cross talk with other signaling pathways contributes to context-specific responses, (iii) the identification of *bona fide* substrates that are responsible for specific function, and (iv) the physiological and pathological roles of MAPKAPK family members.

The application of systems biology approaches and high-throughput genomic and proteomic techniques may provide valuable insights into these important questions. Moreover, the use of genetically modified mice to modulate expression of the MAPKAPKs in a time- and tissue-specific manner will be very useful to elucidate *in vivo* functions. The identification of MK5 as a potential mediator of oncogene-induced senescence or of MK2 as an essential mediator of the inflammatory response underscores the fact that the generated knowledge may be translatable into new therapeutic opportunities. It is likely that we are just beginning to understand the cellular processes regulated by the MAPKAPKs, and future studies should be most enlightening.

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