Phosphatase-mediated crosstalk between MAPK signaling pathways in the regulation of cell survival

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ABSTRACT **Mitogen-activated protein kinase (MAPK) pathways constitute a large modular network that regulates a variety of physiological processes, such as cell growth, differentiation, and apoptotic cell death. The function of the ERK pathway has been depicted as survival-promoting, in essence by opposing the proapoptotic activity of the stress-activated c-Jun NH_2-terminal kinase (JNK)/p38 MAPK pathways. However, recently published work suggests that extracellular regulated kinase (ERK) pathway activity is suppressed by JNK/p38 kinases during apoptosis induction. In this review, we will summarize the current knowledge about JNK/p38-mediated mechanisms that negatively regulate the ERK pathway. In particular, we will focus on phosphatases (PP2A, MKPs) as inhibitors of ERK pathway activity in regulating apoptosis. A model proposed in this review places the negative regulation of the ERK pathway in a central position for the cellular decision-making process that determines whether cells will live or die in response to apoptosis-promoting signals. In addition, we will discuss the potential functional relevance of negative regulation of ERK pathway activity, for physiological and pathological conditions (*e.g.*, cellular transformation).—Junttila, M.R., Li, S.-P., Westermarck, J. Phosphatase-mediated crosstalk between MAPK signaling pathways in the regulation of cell survival. *FASEB J.* 22, 954–965 (2008)**

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Mitogen-activated protein kinase (MAPK) pathways constitute a large kinase network that regulates a variety of physiological processes, such as cell growth, differentiation, and apoptotic cell death. However, deregulation of MAPK activity has been implicated in several pathological situations, including inflammation, oncogenic transformation, and tumor cell invasion. To date, three MAPK pathways have been characterized in detail (**Fig. 1**). The ERK pathway is activated by a large variety of mitogens and by phorbol esters, whereas the c-Jun NH_2-terminal kinase (JNK)/stress-activated protein kinase (SAPK) and p38 pathways are stimulated mainly by environmental stress and inflammatory cytokines (1–5) (Fig. 1). In addition, other less well-characterized MAPK pathways exist, such as the extracellular regulated kinase 5 (ERK5) pathway (6) (Fig. 1). MAPK cascades are organized as modular pathways in which activation of upstream kinases by cell surface receptors leads to sequential activation of a MAPK module (MAPKKK → MAPKK → MAPK) (Fig. 1). After MAPKs (ERK1, 2, JNK1–3, and p38α,β,γ,δ) are activated either in the cytoplasm or in the nucleus, they bind and regulate transcription by modulating the function of a target transcription factor through serine/threonine (ser/thr) phosphorylation (1–5) (Figs. 1 and **2*B***). In addition to the transcriptional effects of MAPK signaling, accumulating evidence indicates that MAPKs regulate cell behavior also by phosphorylating cytoplasmic target proteins, such as apoptotic (*e.g.*, BH3-only family) or cytoskeletal proteins. Several comprehensive reviews of MAPK pathways have been published, and the reader is encouraged to become acquainted with these for detailed information about the structure, function, and biochemistry of MAPK signaling (1–5). This review focuses on emerging information that describes the mechanisms and functional significance surrounding the negative regulation of ERK pathway signaling by stress-activated JNK/p38 pathways.

EXTRACELLULAR-REGULATED KINASE 1,2 (ERK) PATHWAY

The ERK pathway (A-Raf, B-Raf, Raf-1→MEK1, 2→ERK1,2) is activated mainly in response to mitogens and growth factors. This pathway has long been associated with cell growth, cell proliferation, and survival (7–9). Most of the signals activating the ERK pathway are initiated through receptor-mediated activation of the small G-protein, Ras (10) (Fig. 2*B*). Ras is a membrane-bound protein activated through the exchange of bound GDP to GTP. The process of activating Ras, thereby requires the recruitment of proteins responsible for initiating GDP/GTP exchange to the membrane, such as SOS (son of sevenless). Activated Ras,

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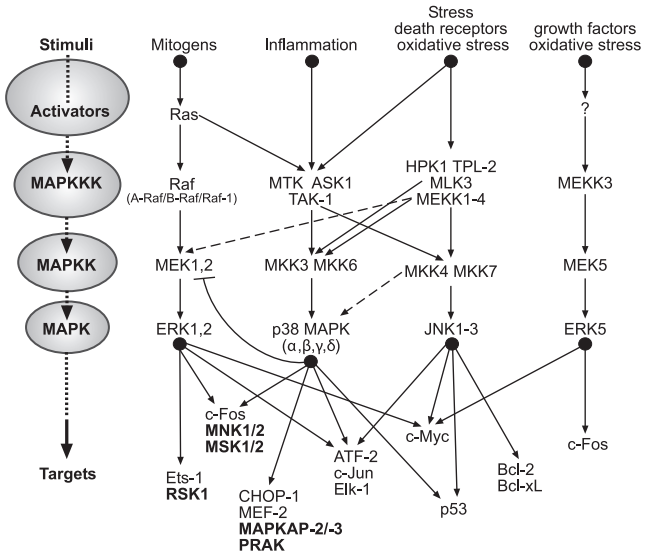

Figure 1. MAPK signaling pathways. MAPK signaling pathways are organized in modular cascades in which activation of upstream kinases by cell surface receptors lead to sequential activation of a MAPK module (MAPKKK → MAPKK → MAPK). Shown are the major MAPK pathway components and examples of the MAPK pathway target proteins. Target kinases are in bold. Dotted lines indicate context-dependent signaling connections between MAPK modules.

then recruits cytoplasmic Raf (MAPKKK) to the cell membrane for activation. There are three mammalian serine/threonine Raf kinases: A-Raf, B-Raf, and Raf-1 (also known as C-Raf). According to gene deletion studies in mice, these proteins have distinct biological functions (reviewed in ref. 11). All three Raf proteins share the same downstream MAPKK substrate mitogen-activated protein kinase kinases 1,2 (MEK1,2).

MEK1,2 is activated by dual phosphorylation on two serine residues by Raf proteins. In addition, recent studies have demonstrated evidence for Ras/Raf-independent activation of MEK1,2 by both p21 kinase (PAK) and MEKK1–3 kinases (12–16). MEK1 and MEK2 are dual-specificity kinases, which share 80% amino acid sequence identity. ERK1,2 is activated by MEK1,2, specifically by phosphorylating a tyrosine and a threonine residue, separated by a glutamate residue (TEY) within the activation loop of the ERK protein (4, 17). ERK1 and ERK2 share 85% amino acid identity and are ubiquitously expressed. Activated ERK1,2 can translocate to the nucleus, where it activates several transcription factors, such as c-Fos, ATF-2, Elk-1, c-Jun, c-Myc, and Ets-1 (Fig. 1). Activated ERK1,2 can also phosphorylate cytoplasmic and nuclear kinases, for example MNK1, MNK2, MAPKAP-2, RSK, and MSK1,2 (4, 17).

In mouse fibroblasts, serum-elicited ERK1,2 activation was originally shown to be required for proliferation and transformation (18). Moreover, in human fibroblasts and mammary epithelial cells, Ras-mediated activation of Raf was identified as one of the requirements for transformation (19). In addition, mutations of B-Raf, which increase the activity of the MEK1,2-ERK1,2 pathway, were found in several malignancies and expression of such mutants in NIH3T3 cells lead to transformation (11, 20). In particular, the B-Raf mutation V600E was detected in ~70% of malignant melanomas, strongly supporting a positive role for ERK pathway activation in melanoma progression (20, 21).

In addition to proliferation, ERK1,2-mediated signaling also has a crucial role mediating cell survival. For example, activated alleles of MEK1 and MEK2 promote cell survival independently of survival factors. Moreover, dominant interfering mutants of MEK1 and MEK2 alleles disrupt cell survival signaling (reviewed in refs. 7, 8). ERK1,2-mediated survival signaling has been proposed to be mediated mainly through activation of RSK kinase (7, 22). Activated RSK phosphorylates, and thereby inactivates, the proapoptotic protein BAD. RSK can also activate the transcription factor CREB, which promotes cell survival through transcriptional up-regulation of antiapoptotic Bcl-2, Bcl-xL, and Bcl-1 proteins (7, 22). In addition, ERK1,2 activity can suppress Fas-mediated apoptosis by inhibiting the formation of the death-inducing signaling complex (DISC) (23).

The utility of MEK-ERK pathway inhibition in cancer therapy was originally demonstrated by suppression of colon tumor growth in a mouse model by chemical inhibition of MEK1,2 (24). Several other studies, including work by Rosen and collaborators using chemical inhibition of the B-Raf V600E chaperone Hsp90, have further validated MEK-ERK pathway inhibition as an attractive opportunity for cancer therapy (21, 25, 26).

c-JUN N-TERMINAL KINASE (JNK) PATHWAY

The JNK (c-Jun N-terminal kinase) pathway is mainly activated by cellular stress and by cytokines. These stimuli activate JNKs through several upstream kinases (MAPKKKs), such as ASK1, HPK1, MLK-3, MKKK1–4, TAK-1, and TPL-2 (2, 5) (Fig. 2*B*). MAPKKs for JNKs are MKK4 and MKK7, which are both needed to fully activate JNK. Both MKK4−/− and MKK7−/− mice are embryonic lethal (27, 28), but cell culture experiments using fibroblasts derived from MKK4 and MKK7 knockout mice revealed that MKK7 mediates JNK inflammatory responses, and both MKK4 and MKK7 are needed for stress-induced JNK activation (28). Interestingly, a recent study provided evidence of an alternative pathway for JNK activation, through reactive oxygen-mediated suppression of JNK phosphatase activity (29).

Three JNK genes—*JNK-1*, *JNK-2*, and *JNK-3*—are susceptible to alternative splicing, resulting in more than 10 JNK isoforms (2, 5). Like all other MAPKs, JNKs are activated through phosphorylation of a tyrosine and a threonine residue, although specificity from the other MAPKs is ensured by the separating proline (TPY) within the activation loop of the kinase. JNKs share 85% sequence identity and are expressed ubiquitously.

JNK pathway activity can mediate apoptosis, proliferation, or survival, depending on the stimuli and cellular conditions. Interestingly, sustained JNK activity is necessary for cellular homeostasis, whereas strong stress

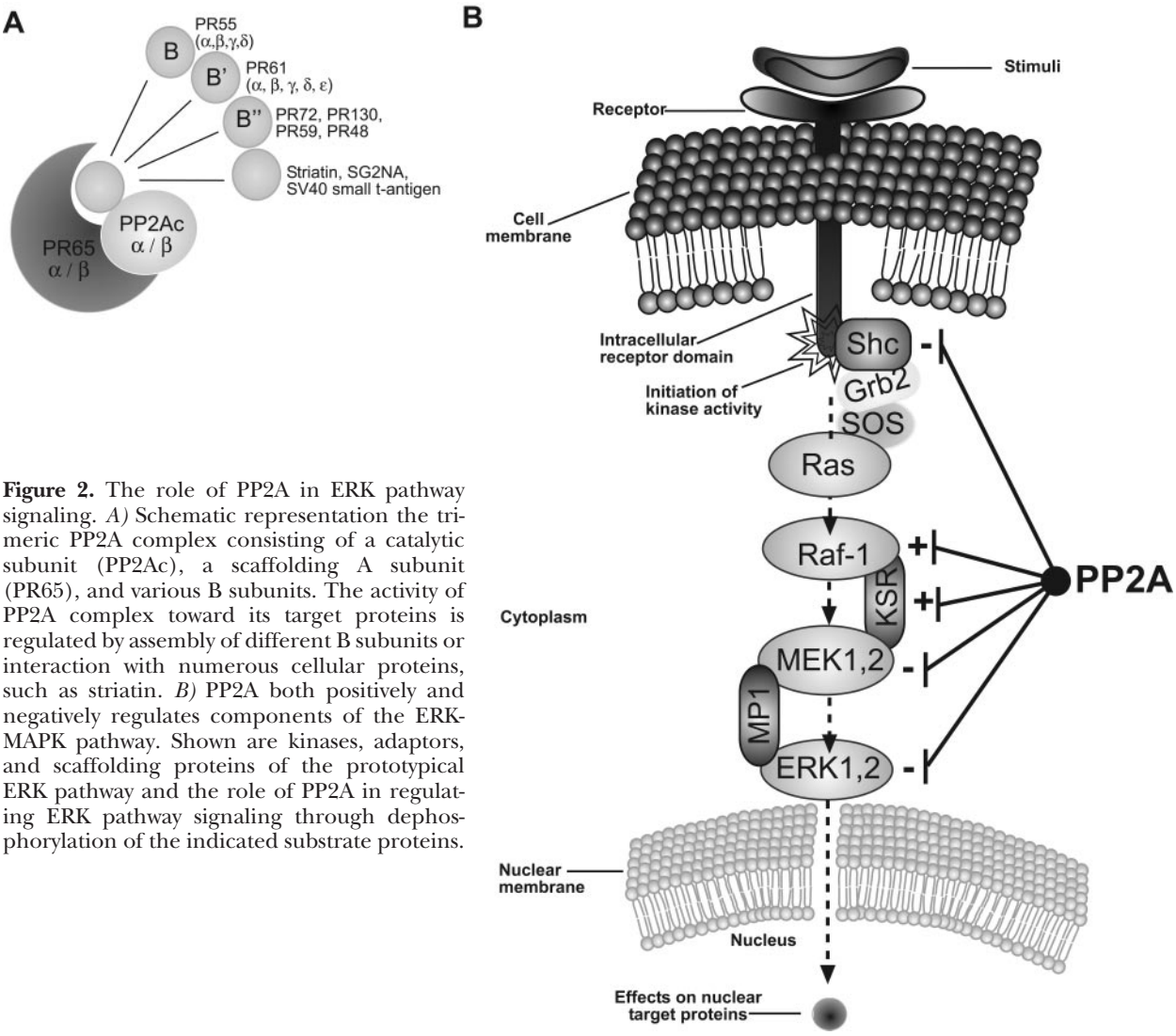

Figure 2. The role of PP2A in ERK pathway signaling. *A)* Schematic representation the trimeric PP2A complex consisting of a catalytic subunit (PP2Ac), a scaffolding A subunit (PR65), and various B subunits. The activity of PP2A complex toward its target proteins is regulated by assembly of different B subunits or interaction with numerous cellular proteins, such as striatin. *B)* PP2A both positively and negatively regulates components of the ERK-MAPK pathway. Shown are kinases, adaptors, and scaffolding proteins of the prototypical ERK pathway and the role of PP2A in regulating ERK pathway signaling through dephosphorylation of the indicated substrate proteins.

stimuli in nontransformed cells primarily leads to JNK-mediated apoptosis (2, 5). In knockout mouse models, the removal of any JNK isoform alone resulted in healthy and viable offspring, although some T cell abnormalities were observed in JNK1−/− and JNK2−/− mice (2, 5, 30). Double knockout mice, lacking both JNK1 and JNK2, were embryonic lethal due to altered apoptosis during brain development (5, 30, 31). JNK3−/− mice demonstrated differences in neuronal apoptosis as compared to wild-type mice (32). Together these findings demonstrate specific functional differences between the JNK isoforms.

The most classical JNK substrate is the transcription factor c-Jun, from which JNK derived its name (2, 5, 33). JNK can activate other transcription factors, such as ATF-2, Elk-1, MEF-2c, p53, and c-Myc. JNK also has other nontranscriptional substrates, for example the antiapoptotic proteins, Bcl-2 and Bcl-xL (2, 5, 34).

p38 PATHWAY

The p38 MAPK pathway (MAPKKKs/MKK3,4,6/p38α,β,γ,δ) can be activated in response to a plethora of inflammatory cytokines, as well as pathogens and by environmental stress, such as osmotic stress, ultraviolet light, heat shock, and hypoxia. It can also be activated by some mitogens, including erythropoietin, colony stimulating growth factor 1, and granulocyte macrophage colony stimulating factor (reviewed in refs. 4, 35). When considering the diverse range of signals that activate the p38 MAPK pathway, it is not surprising that several MAPKKKs can initiate the p38 MAPK signaling module and that the specificity of activation may be determined by the stimuli (Fig. 2*B*). For example, MTK1 cannot mediate cytokine signaling but can only stress signaling (4, 35). Moreover, nonredundant functions have been attributed to p38 MAPKKs, MKK3, and MKK6, *in vivo* (36–38). For example, it was shown that during T cell apoptosis MKK3 activity is required but not that of MKK6 (39). In addition to MKK3 and MKK6, activation of p38 has been reported for MKK4 *in vitro* (40) and *in vivo* (41, 42).

The p38 MAPK protein is represented by four isoforms: p38α, p38β, p38γ, and p38δ. Activation of all the p38 isoforms is achieved by dual phosphorylation of a threonine and a tyrosine within the threonine-glycine-

tyrosine (TGY) sequence in the activation domain of the kinase (4, 35, 43). Phosphorylated p38 proteins can activate an array of transcription factors, including ATF-2, CHOP-1, MEF-2, p53, and Elk-1. Importantly, p38 can also activate other kinases, such as MNK1 and MNK2, MSK1, PRAK, MAPKAPK-2, and MAPKAPK-3.

Activation of the p38 MAPK pathway is required for apoptosis induction in several different cellular models (38, 39, 44–48). Additionally, stress-elicited p38 activation was shown to cause G2/M cell cycle arrest and to regulate the cell cycle through modulation of p53 and p73 tumor suppressor proteins (49, 50). Conversely, p38 MAPK pathway activity has been reported to promote cancer cell growth and survival. For instance, high p38 MAPK activation has been observed in some cancer types, as compared to their matched controls (51–53). p38 MAPK activity also correlated with the invasiveness of several cancer cell lines and inhibition of p38 activity reduced their proliferation, survival, and invasion (53, 54). The molecular mechanisms that determine whether p38 signaling either promotes or inhibits cell proliferation and survival have not been elucidated but could potentially be linked to the transformation state of the cell or could depend on the nature of p38-activating signal. In addition, the p38 pathway plays an essential role in regulating the expression of many inflammatory molecules, differentiation of epidermal keratinocytes, myoblasts, and immune cells, as well as mediates innate immune responses (4, 35, 55).

SPECIFICITY OF MAPK SIGNALING

Specificity of MAPK signaling is maintained primarily through structural mechanisms that limit protein interactions. As described above, all three main MAPKs—ERK, JNK, and p38—contain a specific sequence in their activation loop (TEY, TPY, and TGY, respectively) that is recognized by the MAPKK of the pathway. In turn, MAPKs only efficiently phosphorylate the consensus motif S/TP in their target proteins. In addition to specific phosphorylation motifs in both MAPKs and their substrates, another level of specificity is ensured by conserved docking domains. These domains form a binding site for the kinase and are required for phosphorylation of the substrate (56). It has been shown that docking interactions between MAPK and their substrates are necessary for signaling and that docking site structures can influence pathway-specific input and output (57, 58).

Activation of MAPK cascades usually takes place in multiprotein complexes that contain both upstream and downstream effectors of the given pathway. In these complexes, components of the signaling pathways are tethered together by structural scaffold proteins that provide specific binding sites for each component of the pathway (reviewed in refs. 59, 60) (Fig. 2*B*). Examples of mammalian MAPK scaffolds are kinase suppressor of Ras (KSR), MEK binding protein (MP1), IQGAP1, and c-Jun N-terminal kinase interacting protein-1 (JIP-1) (59–63). Scaffolds are thought to facilitate the spatial concentration of pathway components to ensure effective signal transmission between kinases. However, scaffolds are not static structural proteins, they too are susceptible to posttranslational modifications that can affect their function. One such example would be the ERK1,2-mediated phosphorylation of its scaffold IQGAP, which leads to an increase in MEK1,2-IQGAP complex formation and subsequent further activation of the ERK1,2 protein (63).

Traditionally, MAPK pathways have been depicted as linear signaling pathways with scaffold proteins representing an important mechanism of insulating the pathways from each other. It has been suggested that in normal physiological contexts, MAPK cascades would function independently with no crosstalk between them, whereas interplay between pathways would be induced only during pathological situations when signal strength exceeds the capacity of the pathway (64). The linear model of MAPK signaling has been mostly supported by studies in yeast and drosophila (64). However, it is conceivable that mechanisms facilitating crosstalk between MAPK pathways in mammalian cells could have evolved to increase the possibilities of specific cellular responses without increasing the amount of components in the pathways. This theory is exemplified by findings that, in contrast to the Raf-MEK-ERK pathway, MAPKKKs for JNK and p38, MEKK1–3, have been shown to branch out and activate MEK1,2 by direct phosphorylation (12, 15, 16) (Fig. 1).

REGULATION OF MEK-ERK PATHWAY BY PHOSPHATASES

Reversible phosphorylation of MAPK proteins emphasizes the importance of balance between the phosphorylating kinases and dephosphorylating phosphatases in regulating these pathways. In general, inactivation of signaling proteins is essential for cell physiology in order for cells to remain responsive to stimuli and to prevent deleterious effects of prolonged pathway stimulation. Proper regulation of the dephosphorylation activities regulating MAPK signaling was already recognized when the function of protein kinases was discovered (65); however, the role of phosphatases in the regulation of MAPK signaling is still poorly understood. Interestingly, a computational model outlining the functional relevance of phosphorylation and dephosphorylation reactions of the epidermal growth factor-induced ERK1,2 pathway was recently published (66, 67). Results of these studies suggested that Raf dephosphorylation, MEK1,2 phosphorylation, and MEK1,2 dephosphorylation were the most important reactions controlling the signal propagation through the ERK pathway. Furthermore, it was concluded that collectively, kinases control signal amplitude, whereas phosphatases mediate both signal amplitude and signal duration (66, 67).

All levels of MAPK signaling can be regulated by

protein phosphatases (Fig. 2*B*). ERK pathway phosphatases are classified according to their substrate specificities into dual-specificity MAPK phosphatases (MKPs), protein serine/threonine phosphatases (PSPs), and protein tyrosine phosphatases (PTPs) (for reviews see refs. 65, 68–70). Interestingly, at least one example has been reported where two different families of phosphatases cooperate in complex to regulate ERK1,2 dephosphorylation. Wang and colleagues characterized the cholesterol-dependent assembly of a phosphatase complex, containing both PP2A and HePTP that dephosphorylates both the serine and tyrosine residues in ERK's activation loop (71).

Protein tyrosine phosphatases (PTPs) comprise a very large family of enzymes that dephosphorylate tyrosine residues. A recent analysis identified 107 genes in the human genome that encode members of four PTP families (69). The Class I cysteine-based family of PTPs is by far the largest of the PTP families with 99 members, which are further classified into subfamilies based on protein domain architecture (69). PTPs inhibit ERK pathway activation in part by dephosphorylating receptor tyrosine kinases (RTK) receptors, such as epidermal growth factor receptor or platelet-derived growth factor receptor (72, 73). In turn, Benjamin Neel and co-workers published important evidence for the role of SHP-2 PTP in RTK-mediated ERK1,2 activation through Src-kinases (74). At the MAPK level, hematopoeitic PTP (HePTP) was shown to maintain ERK1,2 in a dephosphorylated state. On phosphorylation of HePTP by protein kinase A, HePTP released ERK1,2, which caused an increase of ERK1,2 activity (75). In a neuronal cell culture model, STEP (a HEPTP homologue) was demonstrated to regulate the duration of ERK signaling in response to *N*-methyl-D-aspartate (NMDA) receptor stimulation (76). Moreover, PTP epsilon was identified as a physiological inhibitor of ERK signaling by protecting cells from prolonged ERK1,2 activation in the cytosol (77).

MAPK phosphatases (MKPs) are a subclass of protein tyrosine phosphatases that can dephosphorylate both phosphotyrosine and phosphothreonine residues on MAPKs (70). Certain MKP family members are more selective for inactivating distinct MAPKs due to an amino terminus kinase-binding domain (70). Regulation of MKP transcription and activity by MAPKs is particularly intriguing because it indicates a complex double-feedback mechanism for mediating phosphatase function (78–80). For example, MKP-1 and MKP-2 expression can be transcriptionally induced with low levels of serum in quiescent fibroblasts, which correlates with subsequent ERK inactivation (78). Activated ERK can also phosphorylate MKP-1 and prevent its degradation by inhibiting ubiquitination (79). In addition, the p38 MAPK target, ATF2, stimulates expression of MKP-1 and thereby inactivates p38 MAPK signaling (81). Moreover, the authors demonstrated that this negative feedback mechanism is essential for survival of embryonic liver cells (81). Such examples emphasize how the balance between phosphatase and kinase activity is critical for proper cellular signaling and that, depending on cellular conditions, MKP-1 can function as either an ERK1,2 or p38 phosphatase.

Protein serine/threonine phosphatases (PSPs) are characterized by their ability to remove phosphate groups from the phosphorylated Ser/Thr residue of a substrate. The majority of PSPs include the type-1 family of phosphatases (PP1) and the type-2 family phosphatases (PP2). The protein phosphatase 2A (PP2A) holoenzyme is a heterotrimer composed of a scaffold (A) and a catalytic subunit (PP2Ac) that associates with a variety of regulatory B subunits (Fig. 2*A*) (68, 82). Several mechanisms, such as holoenzyme composition, methylation of the catalytic subunit, and phosphorylation of the catalytic subunit can regulate PP2A activity. Recent information regarding PP2A holoenzyme structure further illustrates how critical the interplay between holoenzyme components and methylation status of the PP2Ac C-terminal tail is in regulating PP2A function (83, 84). However, the exact mechanism by which methylation of the C-terminal tail of PP2Ac regulates PP2A activity is currently under debate (84–86).

PP2A activity is inhibited by a diverse array of natural toxins, such as okadaic acid (OA) and calyculin A, and by several viral proteins, such as simian virus 40 (SV40) small-t antigen and adenovirus E4orf4 protein (68, 87). Depending on the holoenzyme composition, different PP2A trimers can negatively regulate activity of all MAPK pathways. As an example, it was recently shown that PP2A regulates p38 pathway by association and dephosphorylation of the upstream activator of p38, MKK3 (88). *In vitro* studies indicate that PP2A can dephosphorylate and inactivate both MEK1,2 and ERK1,2 proteins (89) (Fig. 2*B*). In addition, known PP2A inhibitors, such as OA and SV40 small-t-antigen, also induce MEK1,2 and ERK1,2 phosphorylation and activity *in vitro* (89, 90). Moreover, transgenic overexpression of a dominant-negative form of the PP2A catalytic subunit in mouse brain caused increased MEK1,2 phosphorylation, providing *in vivo* evidence for the negative regulation of MEK1,2 by PP2A (91).

Studies have further delineated PP2A's regulation of the ERK1,2 pathway by investigating the differences between heterotrimeric PP2A complexes. For instance, in drosophila S2 cells, PP2A-containing B56 specifically inhibited ERK1,2 phosphorylation (92). Moreover, in the human sympathetic neuron cell line, PC6–3, overexpression of the PP2A regulatory subunit, B55γ, induced ERK pathway activity, whereas B55α/δ directly promoted ERK1,2 dephosphorylation (93, 94). Even though the studies described above provided important information about the identity of the B-subunits responsible for PP2A recruitment to ERK1,2, the B-subunits specific for MEK1,2 have yet to be determined. Interestingly, growth factor induction of the early response gene IEX-1, a known substrate of ERK1,2, mediated the interaction of ERK1,2 with B65-containing PP2A (95). ERK1,2-mediated phosphorylation of B56 caused its dissociation from the PP2A trimer, thereby protecting ERK1,2 from dephosphorylation by PP2A (95). Along the same lines, association of the MEK-ERK scaffold, IQGAP1, with E-cadherin was re-

cently shown to be regulated by PP2A activity (63, 96). These results demonstrate that the effects of PP2A activity on the MEK-ERK signaling module can be mediated by modulating their scaffold proteins.

PP2A can also regulate ERK pathway signaling by interacting with upstream activator proteins (Fig. 2*B*). The adapter protein Shc participates in receptor-mediated activation of Ras. PP2A was shown to bind Shc, thereby preventing its tyrosine phosphorylation and inhibiting the signal progression (97). Earlier studies in drosophila suggested that PP2A negatively regulates signaling from Ras to Raf-1 but positively mediates signaling from Raf-1 (98). Moreover, Raf-1 was shown to interact with PP2A, possibly resulting in Raf-1 activation (99). A positive role for PP2A in Ras-mediated signaling can be explained by PP2A-mediated dephosphorylation of critical sites on both the scaffold proteins, KSR and Raf-1, facilitating their recruitment to the membrane (59, 100). Similarly, PP2A, along with the prolyl isomerase Pin1, were shown to cooperate in dephosphorylating Raf-1 on critical inhibitory sites that prevent Raf-1 reactivation (101). Additionally, dephosphorylation of serine 985 on hepatocyte growth factor receptor c-Met, was shown to stimulate receptor responsiveness and down-stream signaling activation (102). Therefore, although the classical role of PP2A in ERK pathway signaling is that of negative regulation, clearly PP2A can also have a positive role on signaling through the pathway.

In addition to the PP2A-class of PSPs, a recent siRNA screen for negative regulators of ERK pathway activity identified PP2C family phosphatase, PPM1α, as a novel inhibitor of ERK activation in human cells (103). This is an important finding, as PP2C-class phosphatases have been traditionally linked to negative regulation of the stress-activated p38 MAPK pathway (104).

INHIBITION OF THE ERK PATHWAY BY STRESS-ACTIVATED MAPK SIGNALING

Inhibition of the ERK pathway by p38 signaling

In nontransformed cells, phosphorylated MEK1,2 is continuously dephosphorylated by PP2A. The constitutive activity of PP2A is stimulated by at least two kinases: p38 MAPK and casein kinase 2 (CK2) (38, 105–108). Inhibition of p38 results in the accumulation of phosphorylated MEK1,2 and ERK1,2, and renders cells resistant to stress-induced MEK1,2 dephosphorylation (38, 106, 107, 109–111). Most of the evidence for accumulation of phosphorylated MEK1,2 and ERK1,2 in response to p38 inhibition has been obtained by using chemical inhibitors of p38 MAPK, such as SB203580. However, we and others have demonstrated negative regulation of the ERK pathway through p38 signaling by expressing dominant-negative components of the p38 pathway, MKK6, p38α, and p38β (38, 109). In both studies, blockade of p38 signaling was shown to prevent the functional outcome of ERK pathway inhibition, namely stress-induced apoptosis and muscle differentiation (38, 109). Importantly, the involvement of PP2A in p38-mediated MEK1,2 dephosphorylation is supported by the observation that p38 activity increases the physical association between endogenous PP2A and the MEK1,2-ERK1,2 complex (47, 107). Moreover, PP2A activity is required for p38-mediated dephosphorylation of MEK1,2 (47, 106, 107, 111) (**Fig. 3*A***). Interestingly, p38/PP2A-mediated MEK1,2 inhibition seems to be a evolutionary conserved process, as a recent study demonstrated function of this mechanism also in rainbow trout fibroblasts in response to anoxia (111).

CK2 directly binds PP2A and also stimulates PP2A activity toward MEK1 *in vitro* (105). *In vivo* experiments utilizing overexpression of CK2 induced MEK1 dephosphorylation and inhibited tumor cell growth and foci formation induced by a constitutively active form of Ras. However, such inhibition was not achieved if cells were transfected with a phosphatase-resistant form of

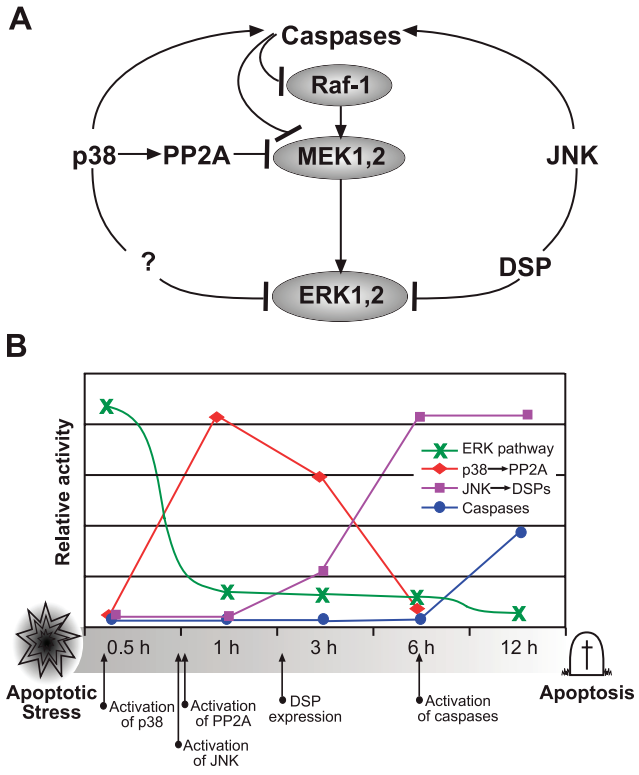

Figure 3. The role of JNK/p38 pathway activation in apoptosis induction through inhibition of the ERK pathway activity. *A)* Summary of the mechanisms negatively regulating ERK pathway activity in response to activation of JNK/p38 signaling. Inhibition of ERK1,2 phosphorylation by direct interaction with p38 is denoted with a question mark since the molecular mechanism of inhibition is unknown. *B)* Proposed model for the induction of apoptosis through inhibition of ERK pathway activity by proapoptotic signaling mediated by the JNK/p38 pathways. In normal cells, activation of p38 signaling causes rapid inactivation of ERK pathway through PP2A activation. This is followed by other indicated mechanisms that ensure shutoff of the ERK pathway finally leading to caspase-mediated cleavage of signaling proteins and apoptosis induction. The indicated timepoints for the signaling events and their functional consequences represent a model based on data from the original citations described in the text.

active MEK1 (105). Interestingly, p38 MAPK has been shown to activate CK2 (112), making it plausible that p38 and CK2-mediated PP2A activation and MEK1,2 dephosphorylation are at least partly the same phenomenon.

In addition to p38→PP2A-mediated MEK1,2 dephosphorylation, direct interaction between p38 isoforms and ERK1,2 has been proposed as a mechanism to inhibit ERK1,2 phosphorylation and activity (48, 55, 113) (Fig. 3*B*). P38α and ERK1,2 were shown to directly interact in a GST-pulldown assay, and evidence was provided for the *in vivo* interaction between transfected proteins in HeLa cells (113). In keratinocytes, endogenous p38δ and ERK1,2 were isolated in complex, and chemical activation of p38δ was associated with inhibition of ERK1,2 phosphorylation (48, 55). However, the molecular mechanism by which direct binding of p38 and ERK1,2 would result in ERK1,2 dephosphorylation was not investigated in these studies. In both models, p38 activation by overexpression of MKK6 prevented ERK1,2 phosphorylation 24–48 h after transfection (48, 55, 113). Because inhibition of ERK1,2 phosphorylation by activated p38 was observed after several hours, it is also possible that ERK1,2 dephosphorylation is mediated by inducing expression of a phosphatase or by some other indirect means. One plausible explanation could be induction of MKP-1 expression through the p38→ATF2 pathway (81). Interestingly, a recent study demonstrated MKP-1-mediated ERK1,2 inactivation in response to radiation-induced Ataxia telangiectasia-mutated (ATM) kinase activation (114). Although not directly demonstrated in their study, the fact that both p38 MAPK and ATM positively regulate ATF2 activity (81, 115) suggests that ATM/p38-ATF2-MKP-1 pathway could be involved in ERK1,2 inactivation in response to radiation.

Inhibition of the ERK pathway by JNK signaling

In addition to p38, several reports have indicated that the JNK pathway also has antagonistic effects on ERK pathway activity (103, 116–118). Transcriptional effects of the JNK pathway are largely mediated by the AP-1 transcription factor complex consisting of c-Jun and c-Fos family proteins (2, 119). Both of the studies by the Gillespie and Tzivion laboratories showed that AP-1-mediated gene expression inhibited ERK1,2 phosphorylation (Fig. 3*A*) (117, 118). Gillespie and colleagues demonstrated that phosphorylation of both MEK1,2 and ERK1,2 was diminished in cells transformed with the oncogenic form of c-Jun (v-Jun) (117). By using constitutively active forms of both Ras and Raf proteins, they found that inhibition of the ERK1,2 pathway occurred both between Ras and Raf, as well as directly on ERK1,2. In addition, the expression of two ERK1,2 dual-specificity phosphatases (MKPs), MKP-1 and MKP-3, was increased in v-Jun transformed cells and treatment of cells with an unspecific inhibitor of MKPs, sodium pervanadate, restored ERK1,2 phosphorylation in v-Jun transformed cells (117). In another study, Tzivion and collaborators showed that forced expression of MLK3, an upstream kinase of both JNK and p38, leads to inhibition of ERK1,2 phosphorylation (118). Furthermore, in this model, AP-1-mediated gene expression seemed to be required for inhibition of ERK phosphorylation, but elevated expression of MKP1–3 in cells expressing active MLK3 was undetectable (118). A role for the JNK pathway in negatively regulating the ERK pathway was greatly strengthened in a recent RNAi screen by the Perrimon laboratory in drosophila S2 cells (103). Their study indicated that the ERK pathway integrates diverse inputs from various cellular processes and that other signaling pathways, such as JNK and Akt/Tor, negatively affect ERK signaling. Moreover, drosophila AP-1 transcription factors D-Jun and D-Fos were specifically identified as negative regulators of ERK pathway activity (103), which is in agreement with the results of Gillespie and Tzivion laboratories (117, 118).

ERK PATHWAY INHIBITION IN REGULATING SURVIVAL AND TRANSFORMATION

As described above, cell survival is dependent on ERK pathway activity in several cellular models, whereas activation of p38 and JNK results in apoptosis induction. The idea that ERK and p38/JNK pathway activities oppose each other as a means of regulating apoptosis was first introduced by Xia and colleagues (46). They demonstrated that NGF withdrawal from differentiated PC12 cells resulted in p38 and JNK activation that preceded ERK1,2 inactivation and apoptosis induction. Moreover, overexpression of constitutively active MEK1 abrogated apoptosis induced by NGF withdrawal (46). However, the mechanisms involved in negatively regulating ERK pathway activity during p38/JNK-mediated apoptosis have only recently been examined.

Regarding p38 signaling, several studies have shown that MEK1,2 dephosphorylation through a p38→PP2A pathway is required for inducing apoptosis in nontransformed cells, such as human skin fibroblasts, cardiac ventricular myocytes, and endothelial cells (38, 47, 107). These conclusions are based on results demonstrating that inhibition of p38 activity either by chemical or genetic means abrogated both MEK1,2 dephosphorylation and apoptosis induction (38, 47, 107). Moreover, overexpression of a constitutively active form of MEK1 that is resistant to PP2A-mediated dephosphorylation resulted in protection from arsenite-elicited apoptosis induction, similar to that observed with inhibition of p38 signaling by dominant negative forms of p38α and β (38). These results are very reminiscent of those reported earlier by Xia *et al.* (46). Also, inhibition of either p38 or PP2A activity inhibited both MEK1,2 dephosphorylation and apoptosis induction in response to H_2O_2 in ventricular myocytes (107). Importantly, PP2A-mediated MEK1,2 dephosphorylation was observed after treating cells with different apoptosis-inducing stimuli, such as arsenite, H_2O_2, TNF-α, and anoxia (38, 47, 106, 107, 111). Together, these results

strongly suggests that proapoptotic stimuli share a general mechanism of preventing survival signaling through PP2A-mediated inhibition of MEK1,2 (Fig. 3).

Interestingly, both activation of ERK1,2 signaling and inhibition of PP2A activity are prerequisites for human cell transformation (19, 87, 120–122). Moreover, inhibition of p38 activity was shown to be required for cellular transformation by activated Raf-1 (123). Importantly, very recent study by Hahn and collaborators demonstrated that, even though both activated B-Raf and MEK1 could promote anchorage independent growth of human cells, only activated MEK1 could support tumor formation in nude mice (122). Therefore, the results described above suggest that the p38/PP2A-mediated MEK1,2 dephosphorylation could be tumor suppressive. In support of this, we have recently demonstrated that activation of p38 signaling results in MEK1,2 dephosphorylation in primary cultures of human fibroblasts and human epithelial keratinocytes, but not in any of the commonly used cancer cell lines or in any low passage head and neck squamous cell carcinoma cell lines derived from human patients (38, 53).

Recent analyses of immortalized and transformed cell lines generated from knockout mouse models for components of p38 pathways did not display increased activity of the ERK pathway (42, 124). More specifically, Nebrada and co-workers were unable to find evidence of p38α-mediated regulation of the ERK pathway in immortalized or transformed mouse embryo fibroblasts (124). The exception being immortalized p38 $-/-$ cardiomyocytes, where p38 pathway signaling suppressed ERK pathway activity (125). Lack of p38-mediated negative regulation of ERK pathway activity in mouse fibroblasts could potentially be explained by inhibition of the p38→MEK crosstalk during cellular immortalization and/or transformation, as suggested by the studies described above, in which malignant counterparts of human primary cells did not display MEK1,2 dephosphorylation in response to p38 activation (38, 53). To confirm whether p38-mediated negative regulation of ERK pathway activity is inhibited during immortalization and/or transformation of mouse fibroblasts, it would be very interesting to further characterize primary mouse cells in this respect.

A MODEL FOR APOPTOSIS INDUCTION THROUGH JNK/p38 AND ERK PATHWAY CROSSTALK IN NORMAL CELLS

The evidence presented in this review demonstrates that the stress-activated JNK and p38 pathways suppress the survival-promoting activity of the ERK pathway. Based on this information, a stepwise model for the regulation of cell survival mediated through JNK/p38 and ERK pathway crosstalk in nonimmortalized cells can be envisioned (Fig. 3*B*). First, the stress-mediated activation of MEK1,2 through MEKK1–3 could be depicted as a signal that promotes cell survival in situations where apoptosis is not yet favorable for the cell (12, 15, 16). However, in circumstances where stress signaling leads to p38-mediated PP2A activation and MEK1,2 dephosphorylation, ERK1,2-mediated survival signaling is inhibited, thereby lowering the threshold for JNK/p38-mediated apoptosis (38, 46, 107) (Fig. 3*B*). In the presence of extended JNK/p38 pathway activation, increased expression of phosphatases (MKPs) that negatively regulate the ERK pathway (81, 103, 114, 117, 118) and an increased physical interaction between p38 and ERK1,2 proteins (48, 113) would secure inhibition of ERK1,2 activity following a transient PP2A activation (Fig. 3*B*). Finally, after apoptosis induction, caspase-mediated cleavage of Raf, MEK, and ERK proteins would represent end-stage removal of survival signaling induced by JNK/p38 signaling (126–129). Altogether, this cascade of signaling events triggered by JNK/p38 activation would shut off ERK pathway-mediated survival signaling and secure stress-induced apoptosis induction in nonimmortalized cells (Fig. 3*B*).

In the future, it would be of great relevance to study the role of these mechanisms, in physiological and pathological conditions *in vivo*. This would be especially important for conditions in which sustained MEK-ERK signaling has been shown to play an important role, such as protection from endothelial cell apoptosis (130, 131) and in T cell differentiation (110, 132, 133). Considering the possible role of negative p38→ERK crosstalk in T cell differentiation, it was recently shown that p38 activity inhibits IL-2 production through inactivation of the ERK pathway (110). Finally, lack of p38-PP2A-mediated MEK1,2 dephosphorylation in human cancer cells (and in immortalized/transformed mouse cell lines) indicates that mechanisms negatively regulating ERK pathway activity could be tumor suppressive (38, 42, 53, 124). In that regard it would also be of great interest to further investigate whether the other ERK pathway inhibiting mechanisms, discussed in this review, would be inactivated during the process of cellular transformation. Information available today, suggests that in contrast to p38, JNK-mediated negative regulation of the ERK pathway also exists in immortalized and transformed cell lines (103, 117, 118). As JNK-mediated mechanisms seem to be dependent on transcriptional activation of gene expression in both human and drosophila cell models (103, 117, 118), they could be considered as homeostasis-regulating mechanisms rather than immediate effectors of ERK pathway inactivation in response to apoptotic insults. However, regardless of the mechanism, it is tempting to speculate that enhancement and/or reactivation of negative regulating mechanisms on the ERK pathway might represent a novel approach for sensitizing cancer cells to therapies (irradiation and chemotherapy) directed to induce stress-mediated apoptosis. Importantly, the role of inhibition of MEK-ERK signaling in apoptosis induction was recently also proposed by Settleman and co-workers, describing ERK1,2 inactiva-

tion following inhibition of the driving oncogenic pathways in cancer cells (134). FJ

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