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3pK, a Novel Mitogen-Activated Protein (MAP) Kinase-Activated Protein Kinase, Is Targeted by Three MAP Kinase Pathways

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Recently we have identified a mitogen-activated protein kinase (MAPK)-activated protein kinase, named 3pK (G. Sithanandam, F. Latif, U. Smola, R. A. Bernal, F.-M. Duh, H. Li, I. Kuzmin, V. Wixler, L. Geil, S. Shresta, P. A. Lloyd, S. Bader, Y. Sekido, K. D. Tartof, V. I. Kashuba, E. R. Zabarovsky, M. Dean, G. Klein, B. Zbar, M. I. Lerman, J. D. Minna, U. R. Rapp, and A. Allikmets, Mol. Cell. Biol. 16:868–876, 1996). In vitro characterization of the kinase revealed that 3pK is activated by ERK. It was further shown that 3pK is phosphorylated in vivo after stimulation of cells with serum. However, the in vivo relevance of this observation in terms of involvement of the Raf/MEK/ERK cascade has not been established. Here we show that 3pK is activated in vivo by the growth inducers serum and tetradecanoyl phorbol acetate in promyelocytic HL60 cells and transiently transfected embryonic kidney 293 cells. Activation of 3pK was Raf dependent and was mediated by the Raf/MEK/ERK kinase cascade. 3pK was also shown to be activated after stress stimulation of cells. In vitro studies with recombinant proteins demonstrate that in addition to ERK, members of other subgroups of the MAPK family, namely, p38RK and Jun-N-terminal kinases/stress-activated protein kinases, were also able to phosphorylate and activate 3pK. Cotransfection experiments as well as the use of a specific inhibitor of p38RK showed that these in vitro upstream activators also function in vivo, identifying 3pK as the first kinase to be activated through all three MAPK cascades. Thus, 3pK is a novel convergence point of different MAPK pathways and could function as an integrative element of signaling in both mitogen and stress responses.

Extensive studies in the last couple of years have identified the Raf/MEK/ERK signaling cascade as a key transducer of signals leading to cell proliferation and differentiation (4, 15, 40, 46). After Raf activation, the signal is transmitted via phosphorylation of the dual-specificity kinase MEK to ERK, which is subsequently translocated to the nucleus to mediate changes in gene expression. Recently two additional parallel kinase cascades composed of enzymes functionally related to either Raf, MEK, or ERK were discovered in mammalian cells (10). These cascades are only poorly activated by mitogens but are strongly stimulated by cellular stress inducers. One of these newly identified cascades is preferentially triggered by anisomycin, UV radiation, and some alkylating agents, and the other is activated by lipopolysaccharide, arsenite, osmotic stress, and heat shock, leading to the activation of JNK/SAPK (Jun-N-terminal kinase/stress-activated protein kinase) (9, 17, 26, 32), and p38RK (MAPKAP-K2 [mitogen-activated protein kinase-activated protein kinase 2] reactivating kinase) (44), respectively. The human homolog of p38RK, CSBP, was independently identified as a kinase involved in the regulation of inflammatory cytokine biosynthesis in human monocytes (30), which was inhibited by the pyridinyl imidazol compound SB203580 (14, 30). SB203580 was found to specifically inhibit p38RK and thus provides a helpful tool to study p38RK-dependent signaling in vivo (14). All three kinases are found in the nucleus upon activation (11, 37), which suggested that they are all distinct members of a family of nuclear shuttle kinases, now commonly designated the mitogen-activated protein kinase (MAPK) family (10). Direct activators of the MAPK family members JNK/SAPK and p38RK are either the dual-specificity kinase SEK1/MKK4 (SAPK/ERK kinase 1) (45), which activates both JNK/SAPK and p38RK when overexpressed (18, 31), or MKK3 (18) and MKK6 (38), which specifically activate p38RK. Originally identified as a MEK1/2 kinase, MEKK1 (28) does not trigger activation of MEK and ERK (57) but stimulates the SAPK activator SEK1 (58) and thus acts parallel to Raf. Since MEKK1 activates SEK1 and JNK but not p38RK in vivo, it is not clear whether SEK1 functions as a physiological p38RK activator (18).

The members of the MAPK family appear to have broad partially overlapping substrate spectra. Thus, the Ets-related transcription factor Elk-1 is phosphorylated by ERK and JNK/SAPK at overlapping sites (55). Another transcription factor, ATF-2, is targeted by all three members of the MAPK family (24, 33, 37, 53). There is also evidence of functionally distinct phosphorylation sites on an overlapping substrate. For example, ERK phosphorylates preferentially the serine residue S-243 in the C-terminal part of c-Jun, which is implicated in a negative regulatory effect (1), whereas JNK phosphorylates the two N-terminal serine residues S-63 and S-73, which potentiates transcriptional activity of c-Jun (36). Other downstream effectors of the MAPK family members include additional kinases, also termed MAPKAP-Ks. RSK/MAPKAP-K1 is activated by ERK in vivo. Initially discovered as a second kinase substrate of ERK (48), MAPKAP-K2 was later identified as a downstream effector of p38RK in arsenite-treated PC12 and A431 cells, in which ERK apparently is not a potent activator of MAPKAP-K2 (44). Consistent with these results, Cano et al. recently reported that MAPKAP-K2 in C3H10T1/2 mouse fi-

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broblasts is not activated by phorbol ester but is activated by anisomycin, indicating that MAPKAP-K2 is a stress-activated rather than a mitogen-responsive kinase (8).

Little is known about the in vivo substrates of RSK/MAPKAP-K1 or MAPKAP-K2 and the physiological effects resulting from their activation. In vitro substrates for MAPKAP-K2 are tyrosine hydroxylase (50) and the murine small heat shock protein Hsp25 and its human counterpart Hsp27 (49). However, only the heat shock proteins could be convincingly demonstrated to be phosphorylated in vivo (14). RSK was originally named for its ability to phosphorylate peptides derived from the C terminus of the ribosomal S6 protein. In vivo RSK phosphorylates the serum response factor, which contributes to regulation of serum response factor-dependent transcription (12). Both kinases phosphorylate glycogen synthase at serine 7. However, studies using a panel of 14 peptides derived from the glycogen synthase N terminus revealed that the substrate preferences of the two kinases are different (16, 48).

Recently we have identified a third MAPKAP-K, which was named 3pK (47). The name is derived from its genetic locus on the short arm of chromosome 3 (chromosome 3p kinase) in a region frequently deleted in small cell lung cancers. Sequence analysis revealed high homology to Ser/Thr kinases, especially to MAPKAP-K2, with sequence identities of 72% on the nucleotide level and 75% on the amino acid level. Besides a potential ERK phosphorylation site C terminal to the kinase domain, the amino acid sequence of 3pK exhibits a putative nuclear localization signal at the C terminus. Initial in vitro studies using immunoprecipitated Raf and purified MEK and ERK proteins showed that 3pK is phosphorylated by ERK after in vitro reconstitution of the kinase cascade (47). Kinase assays with a panel of 14 substrate peptides derived from the N terminus of glycogen synthase (48) revealed that the substrate preference of 3pK is different from those of RSK/MAP KAP-K1 and MAPKAP-K2 (47). Although these in vitro data suggested a function of 3pK in Raf-dependent signaling, the in vivo situation in terms of extracellular stimuli and activating kinases remained unresolved.

MATERIALS AND METHODS

Cell lines, antibodies, and p38RK inhibitor. The human embryonic kidney cell line 293 was cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) (heat inactivated at 56°C for 30 min) at 37°C in humidified air with 5% CO_2. After transfection, cells were starved in 0.3% FCS containing DMEM 48 h prior to stimulation. The human promyelocytic leukemia cell line HL60 was maintained in RPMI 1640 medium supplemented with 10% FCS. A total of 7×10^5 cells per well (2-cm diameter) were seeded in six-well plates and were starved in RPMI 1640 with 0.3% FCS 24 h prior to stimulation. Antisera against bacterially expressed 3pK were raised as described earlier (47). A synthetic peptide corresponding to the C terminus of 3pK (CQAGSSSASQGCNNQ) was covalently coupled to keyhole limpet hemocyanin and used as an immunogen in rabbits. An antibody against p38RK (Mpk-2) was a generous gift from A. Nebreda, Heidelberg, Germany. Anti-glutathione *S*-transferase (GST) antisera were obtained from rabbits immunized with bacterially expressed and purified GST. The specific p38RK inhibitor SB203580 was provided by J. Lee, SKB Pharmaceuticals, King of Prussia, Pa. The inhibitor was used at a concentration of 5 μM from a 20 mM stock solution in dimethyl sulfoxide.

DNA constructs, mutagenesis, and transfection procedures. Mutation of the lysine in the putative ATP-binding site of 3pK [3pK(K→M)] as well as the phosphorylation site mutations 3pK(T→A), 3pK(T→E), and 3pK(TT→EE) were created by a PCR-based oligonucleotide-directed mutagenesis protocol. The cDNAs of 3pK and the mutants were cloned either in the KRSPA eukaryotic expression vector (19), carrying the gene under the control of the Rous sarcoma virus (RSV) promoter, or in the pEBG vector, expressing the gene as a GST fusion protein under the control of the human EF1α promoter (45). Eukaryotic pEBG-SAPKβ and pEBG-SEK1 vectors were a kind gift of J. Kyriakis and L. Zon, Charlestown, Mass. pEBG-SEK1(ST→EE), pEBG-SEK1(K→R), and Hemagglutin (HA) epitope-tagged SAPKβ(KK→RR) were generated by using the Quick Change mutagenesis kit (Stratagene). The pCDNA3-Flag-p38 expression vector was obtained from L. Han, La Jolla, Calif. Raf and Raf mutant expression constructs are described by Bruder et al. (7). The v-Raf expression vector EHneo is described by Rapp et al. (42). Expression plasmids for ERK2 mutants B3 (K52→R) and C3 (Y185→F) have been described previously (43, 52). For transfection of 293 cells, 5×10^5 cells were seeded in a 10-cm-diameter dish and grown for 24 h in DMEM–10% FCS prior to transfection. Transfections were performed by a calcium phosphate coprecipitation method using 5 to 10 μg of DNA unless otherwise indicated, using a modified Stratagene transfection protocol. If cells were cotransfected with different DNAs, DNA content was normalized with an appropriate empty expression vector. Cells were starved in DMEM–0.3% FCS 48 h prior to stimulation. Stimulation of cells was done with 10% FCS in combination with 100 ng of tetradecanoyl phorbol acetate (TPA) per ml, 0.5 mM sodium *meta*-arsenite, or 10 μg of anisomycin per ml for 30 min unless otherwise indicated.

Expression and purification of recombinant proteins. Wild-type and mutant 3pK cDNAs were cloned in a pGEX-KG vector and bacterially expressed as GST fusion proteins essentially according to the Pharmacia protocol. Expression and purification of GST–MAPKAP-K2 and mutants were done as described earlier (20). pGEX-KG-SAPKα and pGEX-KG-c-Jun(1-135) vectors were gifts from J. Kyriakis and L. Zon. All fusion proteins were purified with glutathione-agarose (Pharmacia) and either eluted from the agarose beads with free glutathione [GST-SAPKα, GST–c-Jun(1-135), GST-MAPKAP-K2, and mutants] or cleaved overnight with thrombin (SAPKα, 3pK, and mutants).

Immunoprecipitation and Western blotting (immunoblotting). HL60 cells or transfected 293 cells were lysed in a modified radioimmunoprecipitation buffer (25 mM Tris-HCl [pH 8.0], 137 mM NaCl, 10% [vol/vol] glycerol, 0.1% [vol/vol], 0.1% sodium dodecyl sulfate [SDS], 0.5% [vol/vol] deoxycholate, 1% [vol/vol] Nonidet P-40, 2 mM EDTA, 1 mM Pefabloc, 1 mM sodium vanadate, 5 mM benzamidine, 5 μg of aprotinin per ml, 5 μg of leupeptin per ml) on ice for 30 min. Cell debris was removed by centrifugation at 15,000 rpm for 10 min. Supernatants were then incubated with different antisera for 2 h at 4°C. 3pK was immunoprecipitated with anti-3pK antisera. Tagged versions of proteins were immunopurified with the corresponding antitag antibodies. Immunoprecipitation of p38RK was performed with a rabbit antiserum raised against a C-terminal peptide of *Xenopus* Mpk-2 (44). The immune complexes were precipitated with protein A-agarose and washed extensively with high-salt TLB buffer (20 mM Tris [pH 7.4], 50 mM sodium β-glycerophosphate, 20 mM sodium pyrophosphate, 500 mM NaCl, 10% [vol/vol] glycerol, 1% [vol/vol] Triton X-100, 2 mM EDTA, 1 mM Pefabloc, 1 mM sodium orthovanadate, 5 mM benzamidine, 5 μg of aprotinin per ml, 5 μg of leupeptin per ml). Immunoprecipitates were used for immune-complex kinase assays.

For detection of the proteins in Western blots, the immune complexes were suspended in electrophoresis sample buffer and heated to 100°C for 3 min. After SDS-polyacrylamide gel electrophoresis (PAGE), gels were electroblotted onto polyvinylidene difluoride (PVDF) membranes (Millipore) and subjected to immunodetection using the appropriate primary antibody. Proteins were visualized by using horseradish peroxidase-conjugated antispecies immunoglobulin G antibodies (Boehringer) and a standard enhanced chemiluminescence reaction (Amersham, Little Chalfont, England).

Immune-complex kinase assays. Immunoprecipitated kinases were washed twice, both in high-salt TLB and in specific kinase buffers, and then assayed in the same buffers supplemented with 5 μCi of [γ-^{32}P]ATP, 0.1 mM ATP, and substrate proteins at 30°C for 15 min. 3pK was assayed in 10 mM $MgCl_2$–25 mM β-glycerophosphate–25 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; pH 7.5)–5 mM benzamidine–0.5 mM dithiothreitol–1 mM sodium vanadate, using 5 μg of Hsp27 as a substrate. HA-ERK2 and Flag-p38RK were assayed in the same kinase buffer with either myelin basic protein or 3pK(K→M) as the substrate. Immunoprecipitates of GST-SEK1 or GST-SAPKβ were incubated in 10 mM $MgCl_2$–50 mM Tris-HCl (pH 7.4)–5 mM benzamidine–0.5 mM dithiothreitol–1 mM sodium vanadate. GST-SAPKα was used as a substrate for SEK, and SAPK activity was assayed by phosphorylation of GST–c-Jun(1-135) or 3pK(K→M). Proteins were separated by SDS-PAGE, blotted onto PVDF membranes (Millipore), and detected with a BAS 2000 Bio Imaging Analyzer (Fuji).

Assay for 3pK activity. In vitro kinase assays with bacterially expressed 3pK were performed with equal amounts (1.6 μg) of wild-type and mutant kinases plus 5 μg of Hsp27 as substrates in 3pK kinase buffer at 30°C for 15 min. After gel electrophoresis and blotting onto PVDF membranes (Millipore), Hsp27 phosphorylation was analyzed as described above.

In vitro activation of GST–MAPKAP-K2 and 3pK by p44 MAPK (ERK1) and p38RK. Purified recombinant GST–MAPKAP-K2 and 3pK (1 μM each) were incubated with 5 ng of p44 MAPK (purified from sea star; Biomol) or the anti-Mpk-2 immunoprecipitate for 30 min at 30°C in a kinase reaction mix containing 50 mM β-glycerophosphate, 0.1 mM EDTA, 4 mM magnesium acetate, 0.1 mM ATP, 0.1 μM okadaic acid, and 125 μM sodium vanadate. For experiments analyzing phosphorylation of the enzyme, 1.5 μCi of [γ-^{33}P]ATP was added. Control incubations omitting MAPKs to analyze the influence of autophosphorylation of the recombinant protein were always carried out.

Assay for MAPKAP-K2 activity. Ten-microliter aliquots from the MAPK activation mixture were incubated in a kinase reaction mix (final volume of 25 μl) containing 50 mM β-glycerophosphate, 0.1 mM EDTA, 4 mM magnesium acetate, 0.1 mM ATP, 1.5 μCi of [γ-^{33}P]ATP, and 10 μg of recombinant Hsp25 purified from *Escherichia coli* (22). After 10 min at 30°C, reactions were terminated by adding 8 μl of 4× SDS sample buffer. Proteins were separated by SDS-PAGE. ^{33}P-labeled proteins were detected with a BAS 2000 Bio Imaging

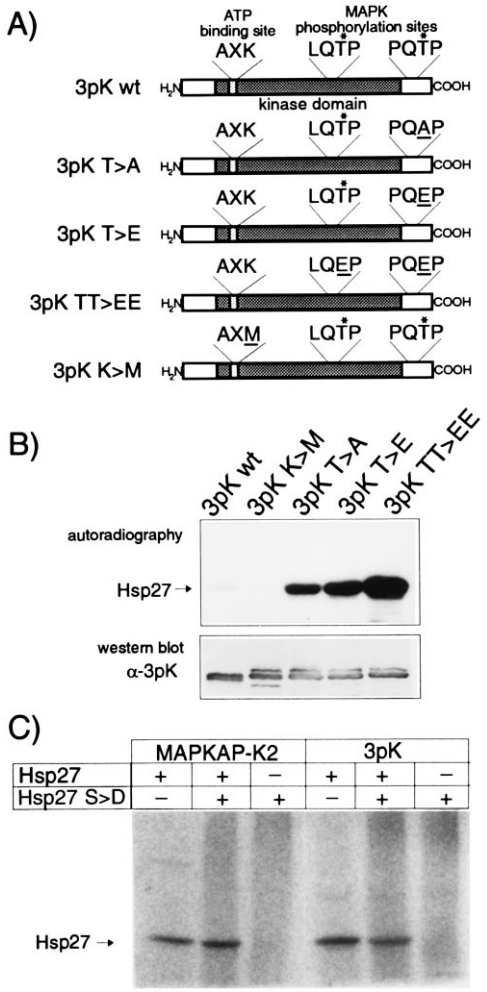

FIG. 1. 3pK is activated by mutation of two potential MAPK phosphorylation sites and phosphorylates Hsp27 at the same sites that are phosphorylated in vivo. (A) Schematic primary structure maps of 3pKwt and the 3pK mutants analyzed. (B) Phosphorylation of Hsp27 by 3pKwt and different mutant proteins. (C) Hsp27 and the phosphorylation site mutant Hsp27 S15,78,82D were phosphorylated by using the constitutively active form of MAPKAP-K2 (GST-MK2-Δ3BΔPC [20]) and 3pK(T→E). A mixture of Hsp27 and Hsp27 S15,78,82D was used to demonstrate that there is no kinase inhibitor in the recombinant Hsp27 S15,78,82D preparation.

Analyzer (Fuji), and Hsp25 labeling was quantified by photo-stimulated luminescence. Assay conditions were tested to guarantee a linear dependence of kinase activity determined on the basis of the assay time chosen.

RESULTS

3pK is constitutively activated by mutation of two potential MAPK phosphorylation sites. The amino acid sequence of 3pK contains a potential ERK phosphorylation site (threonine 313) with the consensus motif PXTP located C terminal to the kinase domain (Fig. 1A). A second sequence matching the consensus motif for phosphorylation by MAPKs is located in kinase subdomain VIII (threonine 201). In MAPKAP-K2, both motifs at analogous positions were shown to be phosphorylated by purified ERK and p38RK (3, 20, 48). We have mutated the threonine at residue 313 of 3pK to glutamic acid [3pK(T→E)] and alanine [3pK(T→A)] (Fig. 1A). A third mutant contains two glutamic acid residues instead of threonine 201 and 313 [3pK(TT→EE)] (Fig. 1A). Kinase activity of these purified recombinant mutants was determined by introducing a novel substrate for 3pK, the human small heat shock protein Hsp27. Hsp25 (murine) and Hsp27 (human) are phosphorylated by MAPKAP-K2 (49), and we found that these proteins are also substrates for 3pK.

The kinase assays revealed that exchange of threonine 313 of 3pK to glutamic acid led to constitutive activation of the kinase, which was approximately increased 14-fold compared to the basal activity of the wild-type protein (3pKwt) (Fig. 1B). Surprisingly, an approximately sevenfold activation was also observed with an alanine in this position, suggesting that the kinase is held in an inactive conformation and any alteration of this site activates the kinase by structural changes. If both threonine 201 and 313 are replaced by an acidic amino acid, mimicking phosphorylation at these sites, the kinase activity is increased to 32-fold compared to the wild-type activity, indicating that a negative charge in both positions is necessary to fully activate the kinase. The fact that both sites involved in activation of 3pK are located within MAPK consensus motifs suggest that 3pK is regulated by phosphorylation through MAPKs. Further, the conserved lysine in the putative ATP-binding domain was replaced by methionine [3pK(K→M)] (Fig. 1A), which completely abolished 3pK kinase activity (Fig. 1B).

3pK phosphorylates the small heat shock protein Hsp27 in vitro at the same sites that are phosphorylated in vivo. It remained to be examined whether 3pK phosphorylates the small heat shock proteins on sites which are found phosphorylated in vivo (23). Hsp27 is phosphorylated on three serine residues (S-15, S-78, and S-82) in conserved amino acid motifs. To address this question, we used Hsp27 S15,78,82D, a phosphorylation site mutant of Hsp27 where the three serine residues had been changed to aspartate (25a). Both active MAPKAP-K2 (GST-MK2-Δ3BΔPC) and active 3pK [3pK(T→E)] phosphorylated the wild-type Hsp27 but were not able to phosphorylate the mutant protein (Fig. 1C). Thus, Hsp27 phosphorylation by 3pK occurs on the sites which are relevant in vivo. Consistent with these results, phosphoamino acid analysis of in vitro-phosphorylated Hsp27 revealed that it is phosphorylated exclusively on serine residues (data not shown).

3pK is activated by serum and TPA in HL60 cells and transiently transfected 293 cells. Since 3pK is phosphorylated in response to serum in vivo (47), we examined whether the kinase is also activated under these conditions. The human promyelocytic cell line HL60 expresses high levels of endogenous 3pK and was therefore chosen for examination of 3pK activation. A significant activation of 3pK was observed after mitogenic stimulation of cells with either 10% serum or 100 ng of TPA per ml (data not shown); however, activation was highest with a combination of both mitogens. HL60 cells were stimulated with serum and TPA for different times. The kinase was immunoprecipitated with a specific antiserum generated against a C-terminal peptide of 3pK. Activity was measured in an in vitro kinase assay using Hsp27 as the substrate. Figure 2A shows that endogenous 3pK in HL60 cells was activated by serum and TPA, with a peak activity after 30 to 60 min. To further study 3pK activation in a different cell system, we introduced 3pK by transient transfection (RSV-3pK) into human embryonic kidney 293 cells, which contain no detectable levels of endogenous 3pK or MAPKAP-K2, as judged by immunoprecipitation and Western blotting. Transfected 3pK could be activated by either serum, TPA, and epidermal growth factor in these cells, and again activation was best with a combination of serum and TPA (see Fig. 5A). Figure 2D shows a time course of 3pK activation by serum and TPA in these cells. A peak activity of approximately 20-fold compared

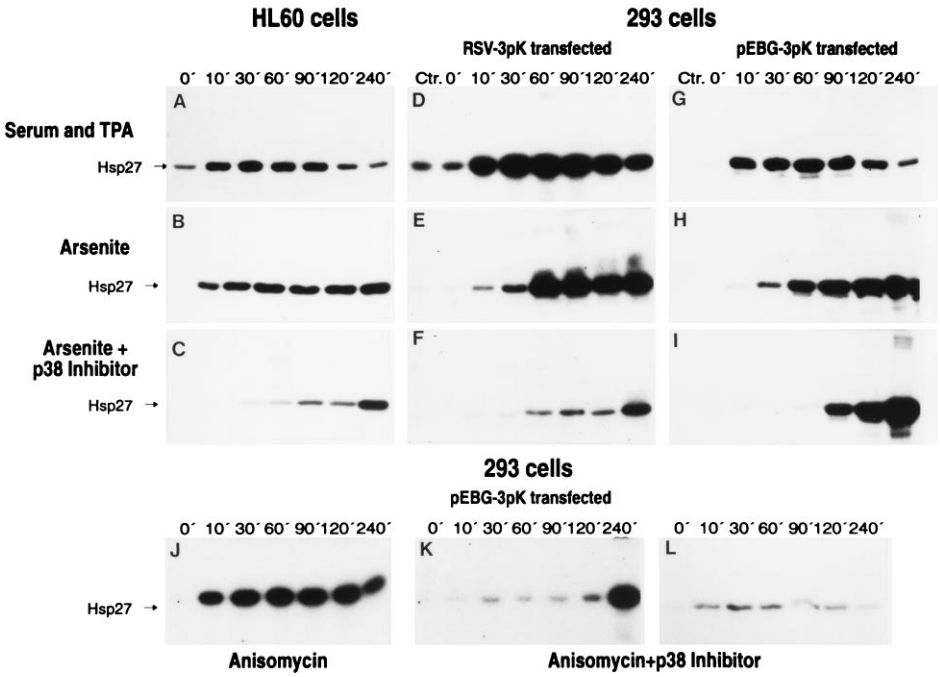

FIG. 2. Activation of 3pK after mitogen and stress stimulation of HL60 cells or transiently transfected 293 cells. HL60 cells were starved in RPMI 1640 containing 0.3% FCS for 20 h and stimulated either with serum and TPA (A) or with arsenite in the presence (C) or absence (B) of the p38RK inhibitor SB203580 for the indicated times (minutes). 293 cells were transfected with 5 μg of RSV-3pK (D to F) or pEBG-3pK (G to L) DNA per dish (10-cm diameter), starved in DMEM containing 0.3% FCS for 48 h, and then stimulated with serum and TPA (D and G), arsenite (E and H), or anisomycin (J) or preincubated with 5 μM SB203580 for 20 min and then stimulated with arsenite (F and I) or anisomycin (K and L) in the presence of the p38 inhibitor for different times as indicated. The experiment shown in panel L differs from that shown in panel K in that the medium was supplied with new aliquots of the inhibitor every 60 min. 3pK activity was assayed after immunoprecipitation with an anti-3pK antiserum (A to F) or an anti-GST antiserum (G to L) in immune-complex kinase assays with Hsp27 as the substrate.

to levels of unstimulated cells was reached after 60 min. To rule out nonspecific effects of the antiserum, the 3pK cDNA was subcloned in a vector expressing the gene as a GST fusion protein (pEBG-3pK), which allows immunoprecipitation with an anti-GST antiserum. The same activation kinetics were observed after transfection and expression of the fusion protein (Fig. 2G).

In vivo activation of 3pK by stress inducers. We further examined whether 3pK is also activated by stimuli other than serum and TPA. Since MAPKAP-K2 is activated by stress inducers, we analyzed whether these stimuli also lead to 3pK activation in vivo. Indeed we found that a variety of stress inducers, including arsenite, anisomycin, tumor necrosis factor alpha, sorbitol, and heat shock, activated 3pK (data not shown). In Figure 2, kinetics of 3pK activation by arsenite in HL60 cells (Fig. 2B) and transiently transfected 293 cells (Fig. 2E and H) are shown. The kinetics of activation as well as the peak activity differed slightly from that observed after serum and TPA activation. Arsenite treatment led to an average of 31-fold stimulation of 3pK kinase activity after 60 min, which, however, does not decrease and is still elevated after 4 h in the presence of the chemical agent. This is not due to an increase in protein amount, as evaluated by Western blotting (data not shown). The same kinetics could be observed after stimulation with anisomycin in both transiently transfected 293 cells (Fig. 2J) and HL60 cells (data not shown).

To analyze which upstream MAPKs are involved in stress-induced 3pK activation, we first used a specific inhibitor of p38RK (SB203580) (14, 30). Activation of 3pK in transiently transfected 293 cells by arsenite could be almost abolished in the presence of the inhibitor (Fig. 2F and I), which is also the case for endogenous 3pK in arsenite-treated HL60 cells (Fig. 2C). Similar results were observed after stimulation of cells with anisomycin (Fig. 2K). The strong inhibition of 3pK activation at early time points clearly shows that p38RK is an upstream activator of 3pK after stress stimulation of cells. Recovery of 3pK activity at late time points (Fig. 2C, I, and K) was due to an instability of the inhibitor, since addition of fresh SB203580 aliquots during the time course of stress stimulation (every 60 min) could prevent recovery of 3pK activity (Fig. 2L).

To rule out that SB203580 inhibits 3pK activity directly, in vitro kinase assays with the constitutively active 3pK mutant in the presence of increasing doses of the inhibitor were performed. For control, the constitutively active mutant of MAP KAP-K2, a kinase which was previously shown not to be inhibited by SB203580 (14), was included. These experiments revealed that 3pK, like MAPKAP-K2, is not affected even at an SB203580 concentration of 100 μM (data not shown).

For further analysis of the specificity of the inhibitor, 3pK activation by serum and TPA or arsenite in the presence and absence of SB203580 was examined. Figures 3A and B show that this reagent could efficiently block 3pK activation after arsenite stimulation, while it did not affect 3pK activity induced by serum and TPA. These findings clearly demonstrate that stress activation of 3pK is mediated by p38RK and potentially other stress-related kinases, while serum and TPA activation of 3pK is accomplished by an independent mechanism.

To test whether the two potential MAPK phosphorylation sites are involved in either stress or mitogen activation of 3pK in vivo, the 3pK(TT→EE) mutant was compared to 3pKwt in transiently transfected 293 cells (Fig. 3C). The basal activity of 3pK(TT→EE) was similar to 3pKwt activity after 60 min of arsenite stimulation, and the mutant could not be further activated by either arsenite or serum-TPA, indicating that 3pK

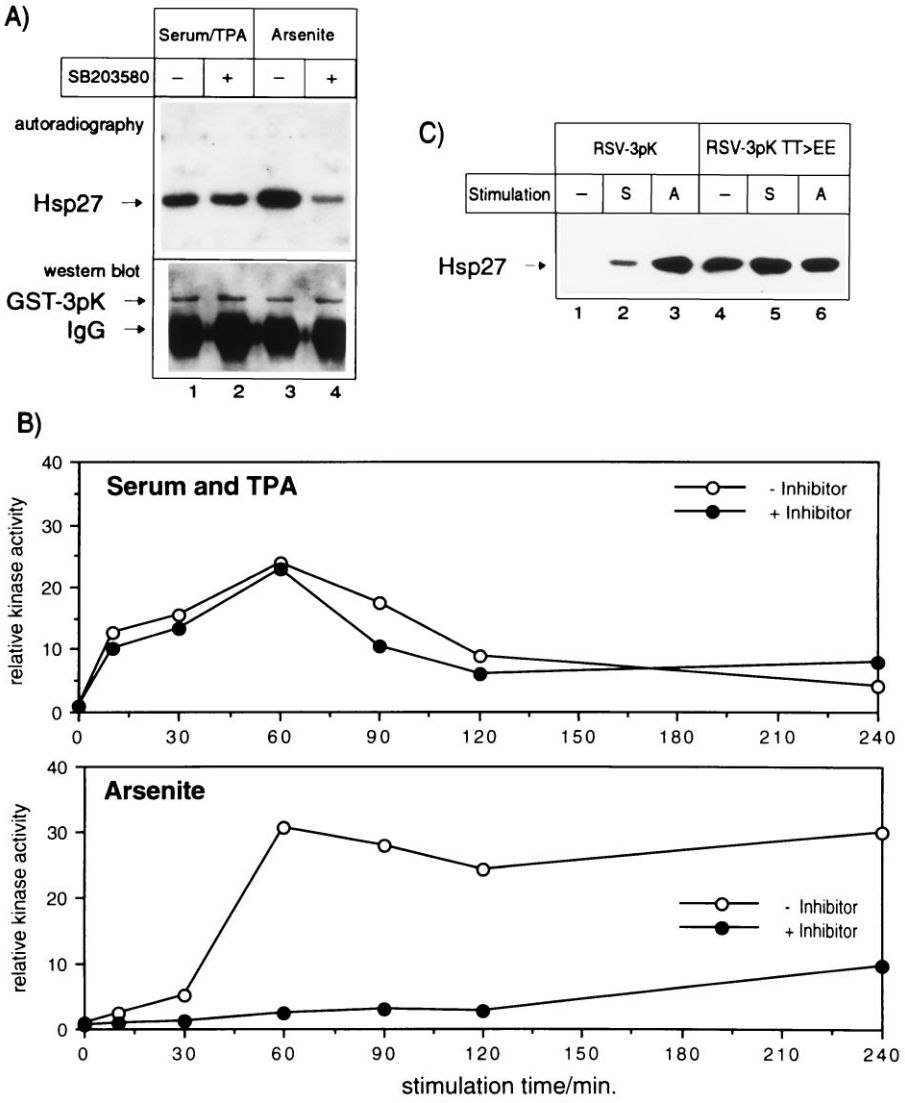

FIG. 3. Selective inhibition of stress-induced 3pK activation by the p38RK inhibitor SB203580. 293 cells were transfected with pEBG-3pK or RSV-3pK and stimulated either with serum and TPA or with arsenite for 30 min (A) or different times (B) in the presence (+) or absence (−) of SB203580 as indicated. (C) Cells were transfected with RSV-3pK or RSV 3pK(TT→EE) and either left unstimulated or stimulated with serum and TPA or arsenite for 60 min. IgG, immunoglobulin G heavy chain.

activation is mediated by phosphorylation of threonines 201 and 313.

3pK is phosphorylated and activated in vitro by ERK and p38RK. To examine which kinases may be involved in mitogen- or stress-induced 3pK activation, in vitro kinase assays with different MAPKs were performed. It was shown earlier that ERK phosphorylates and activates 3pK when assayed with a peptide substrate (47). These experiments were extended with active ERK1 isolated from sea star and 3pKwt together with Hsp25 (Fig. 4A, lane 1) or 3pK(K→M) (data not shown) as the substrate. The results were consistent with the earlier findings. As suggested by the in vivo data for the specific inhibitor, 3pK was also in vitro phosphorylated and activated by p38RK. This could be shown with p38RK either partially purified from anisomycin-stimulated Ehrlich ascites tumor cells (20) (Fig. 4A) or immunoprecipitated from the same cells with an anti-p38 antibody (44) (Fig. 4B). 3pK activation by purified p38RK was as efficient as 3pK activation by sea star ERK and was comparable to the activation of MAPKAP-K2, measured by phosphorylation of Hsp25. Our data are supported by a recent paper by McLaughlin et al. (34) reporting the identification of MAPKAP-K3, which is identical to 3pK, as a downstream substrate of p38RK, both in vitro and in vivo in stress-induced and transiently transfected COS and HeLa cells. In addition to those data, we have shown that this is also true in a physiological context monitoring endogenous kinase activities in HL60 cells (Fig. 2B and C).

3pK is phosphorylated and activated by SAPK in vitro. Anisomycin is known to preferentially activate the JNKs/SAPKs but is also an efficient activator of 3pK in vivo. This suggests that JNKs/SAPKs may also be involved in 3pK activation. Thus, we examined whether SAPK is able to phosphorylate and activate 3pK in vitro. Kinase assays were performed with bacterially expressed active SAPKα (Fig. 4C, lanes 9 to 11), immunoprecipitated GST-SAPKβ (lanes 3 to 6), or recombinant GST-SAPKα activated by immunoprecipitated

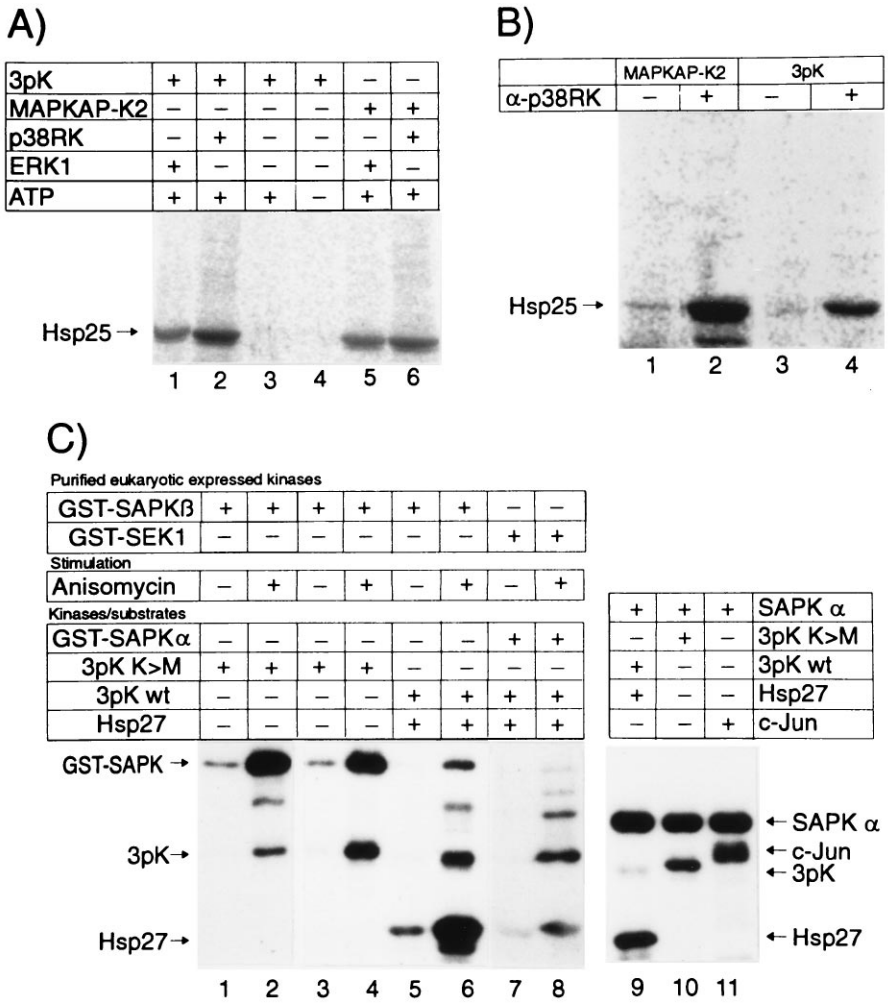

FIG. 4. In vitro phosphorylation and activation of 3pK by ERK, p38RK, and SAPK. (A) Activation of 3pK and MAPKAP-K2 by sea star ERK1 and p38RK which was partially purified from anisomycin-stimulated EAT cells as described previously (20). Activity was assayed by using [γ-^{33}P]ATP and Hsp25 as the substrate. As a negative control and as an assay for autophosphorylation, the enzymes were incubated in the presence of labeled ATP, omitting the activating kinases. (B) Activation of 3pK by immunoprecipitated p38RK. p38RK was immunoprecipitated from EAT cells stimulated with anisomycin (10 μg/ml) for 20 min, using an anti-Mpk-2 antiserum (+) (44). As a control, immunoprecipitation was carried out with rabbit preimmune serum (−). Recombinant 3pK and GST–MAPKAP-K2 were incubated with the immunoprecipitate in the presence of ATP as described above. (C) Phosphorylation of 3pK by SAPK. In vitro kinase assays were performed with either bacterially expressed active SAPKα (lanes 9 to 11) or eukaryote-expressed and in vivo-activated GST-SEK1 (lanes 7 and 8) or GST-SAPKβ (lanes 1 to 6). Activity of recombinant SAPKα is as high as activity of recombinant GST-SAPKα activated by SEK. Eukaryote-expressed SEK and SAPK from anisomycin-stimulated (lanes 2, 4, 6, and 8) or unstimulated (lanes 1, 3, 5, and 7) cells were either immunoprecipitated (lanes 3 to 8) or purified with glutathione-Sepharose (lanes 1 and 2). Substrates were either 3pK(K→M) (lanes 1 to 4 and 10) or 3pKwt together with Hsp27 (lanes 5 to 8 and 9). GST–c-Jun(1-135) was used as a control substrate for SAPK activity (lane 11). Samples containing immunoprecipitated GST-SEK1 were additionally supplied with bacterially expressed GST-SAPKα to in vitro reconstitute the kinase cascade (lanes 7 and 8).

GST-SEK1 (lanes 7 and 8), using 3pK as the substrate. 3pK activity was measured by Hsp27 phosphorylation. In addition, activated GST-SAPKβ was purified from 293 cell lysates (lanes 1 and 2) and used in the same assay. GST–c-Jun(1-135) was used as a control substrate for SAPK activity (lane 11). In all of these experiments, the activated SAPKs were able to phosphorylate and activate 3pK efficiently. Thus, 3pK represents the first kinase which is in vitro phosphorylated and activated by JNKs/SAPKs. The in vitro data suggest that 3pK activation in vivo after stress stimulation may also be mediated to a certain extent by JNKs/SAPKs.

3pK is in vivo activated by mitogen and stress-inducing agents leading to ERK, p38RK, or SAPK activation. For a proper evaluation which MAPK family members are involved in 3pK activation, it was important to monitor the activity of ERK, p38, and SAPK in the cells used. Cells were cotransfected with HA-ERK2-, Flag-p38RK-, and GST-SAPKβ-containing expression vectors and stimulated with mitogen or stress inducers. Figure 5 shows that ERK is activated in 293 cells by serum and TPA and only poorly by stress-inducing agents, whereas the opposite is found for p38RK and SAPKβ. The latter two kinases are highly activated by stress inducers; however, arsenite preferentially activates p38, while anisomycin is a better activator of SAPK. The observation that 3pK is highly activated by either stimulus (Fig. 5A) suggests that all three MAPK family members might be involved in 3pK activation with different preferences depending on the extracellular activator.

Serum- and TPA-induced activation of 3pK in 293 cells is mediated by the Raf/MEK/ERK kinase cascade. To examine whether the Raf/MEK/ERK cascade is involved in 3pK activation by serum and TPA, 293 cells were transiently trans-

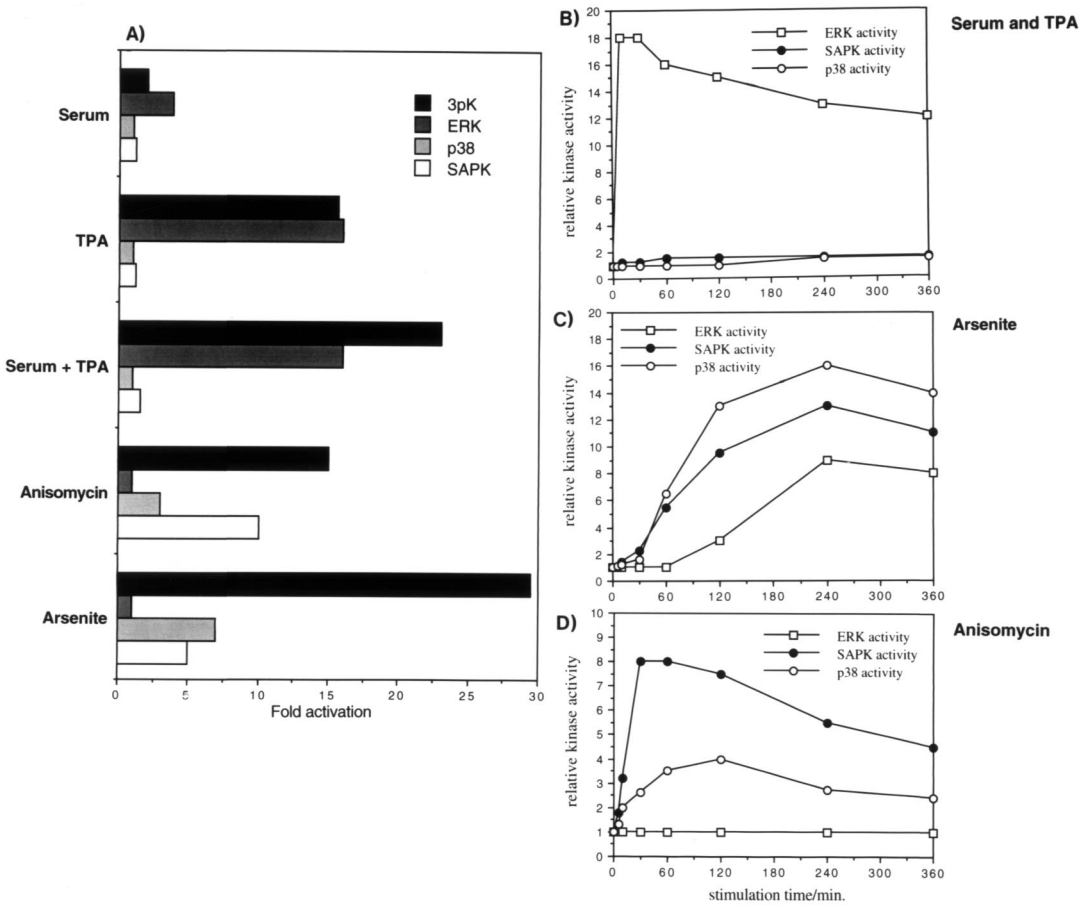

FIG. 5. Immune-complex kinase assays with 3pK, ERK2, p38RK, and SAPKβ. (A) 293 cells were cotransfected with pmt-HA-ERK2 and pEBG-SAPKβ or with pEBG-3pK and pCDNA3-Flag-p38RK and stimulated as indicated for 60 min (30 min with anisomycin). (B to D) Cells were transfected with pmt-HA-ERK2, pEBG-SAPKβ, and pEBG-Flag-p38RK and stimulated with serum and TPA (B), arsenite (C), and anisomycin (D) for different times. Kinases were immunoprecipitated and activities were monitored in a kinase assay using either myelin basic protein, GST–c-Jun(1-135), 3pK(K→M), or Hsp27 as the substrate for ERK, SAPK, p38RK, or 3pK, respectively. Relative kinase activities were calculated on the basis of basal activities in unstimulated cells.

fected with RSV-3pK (Fig. 6A) or pEBG-3pK (Fig. 6B and C) together with expression vectors carrying c-Raf, ERK2, or different constitutively active and dominant negative Raf and ERK2 mutants. RafBXB is a constitutively active mutant consisting of the catalytic (CR3) subdomain of Raf (7). RafC4B consists of the regulatory domain of the Raf protein and has a dominant negative effect on Raf-dependent signaling due to its competitive binding to Ras (7). The ERK2 mutant B3 is an ATP-binding-site mutant (K→52R), while ERKC3 is inactivated by exchange of the tyrosine in the TEY motif to phenylalanine (Y185→F) (43). Both ERK mutants exhibit a basal kinase activity in vitro (≥5% of wild-type activity) (43) but were found to interfere efficiently with Raf-induced transformation of NIH 3T3 cells in vivo (52).

Serum and TPA stimulation of 293 cells transfected with 3pK alone led to an eightfold activation of the immunoprecipitated kinase (Fig. 6A to C, lanes 1 and 2) after 30 min. Coexpression of c-Raf together with 3pK resulted in an enhanced activation of 3pK in the presence of serum, 22-fold compared to the level for unstimulated cells and 2.5-fold compared to the level for stimulated cells transfected with 3pK alone (Fig. 6A and B, lanes 3 and 4). RafBXB coexpression in the absence of serum and TPA activated 3pK 14-fold compared to cells transfected with 3pK alone, mimicking the effect of serum and TPA stimulation (Fig. 6A, lane 5). This effect of RafBXB cotransfection could be also observed in NIH 3T3 cells (data not shown). Coexpression of v-Raf, the oncogenic form of Raf as found in the retrovirus MSV3611 (41), also led to a fivefold activation of 3pK in the absence of serum (data not shown). However, the most dramatic 3pK activation (approximately 50-fold) was observed after coexpression of ERK2 (Fig. 6B, lane 6). To examine whether dominant negative or kinase inactive mutants of Raf and ERK were able to interfere with the serum- and TPA-induced 3pK activation, cells were cotransfected with 3pK and increasing amounts of ERKB3, ERKC3, and RafC4B (Fig. 6C). 3pK activity decreased to 30, 20, and 8% of the wild-type protein activity after cotransfection of B3 (lane 4), C3 (lane 5), and C4B (lane 8), respectively, if the upstream mutant was present in a threefold excess. The ability of ERK2C3 to block 3pK activation more efficiently than ERK2B3 is consistent with previous data on Raf-induced transformation (52).

In summary, these data clearly show that 3pK activation by serum and TPA is indeed Raf dependent and is mediated by the Raf/MEK/ERK kinase module.

Stress-induced activation of 3pK in 293 cells is mediated by p38RK and JNK/SAPK. To evaluate the possibility of 3pK activation by JNK/SAPK in vivo, RSV-3pK was transfected alone or together with pEBG-SEK1 or pEBG-SAPKβ in 293 cells. Cells were treated with anisomycin for 30 min. Coexpres-

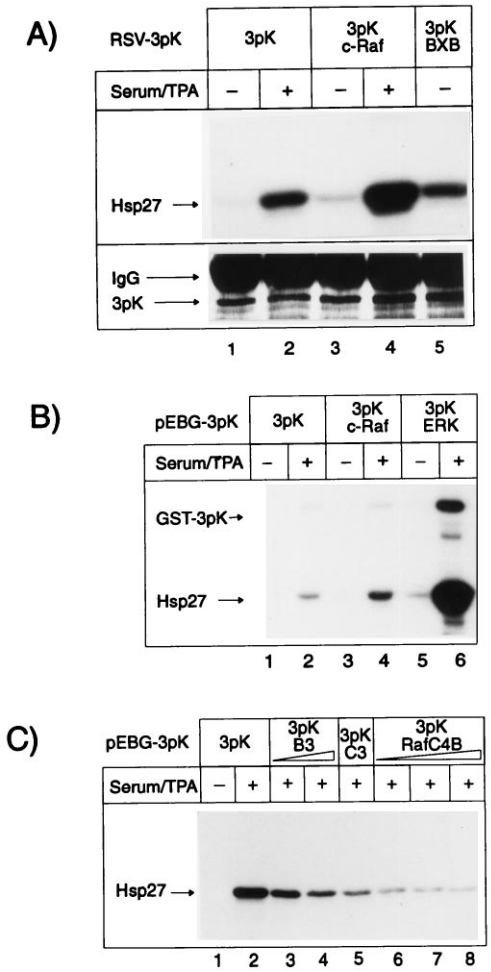

FIG. 6. Cotransfection of 293 cells with 3pK, ERK2, c-Raf, and different Raf and ERK mutants. 239 cells were transfected with 5 μg of each DNA construct per dish. DNA content was normalized by using the corresponding empty vector (KRSPA). Cells were either left unstimulated (−) or stimulated with serum and TPA (+) in the samples indicated. (A) Transfection of RSV-3pK alone or in combination with RSV–c-Raf-1 or RSV-RafBXB as indicated. (B) Transfection of pEBG-3pK alone or in combination with RSV–c-Raf-1 or pmt-HA-ERK2. (C) Transfection of pEBG-3pK alone or in combination with the interfering mutants ERK2B3 in a 1:2 (lane 3) or 1:3 (lane 4) ratio, ERK2C3 in a 1:3 ratio (lane 5), and RSV-RafC4B in a 1:1 (lane 6), 1:2 (lane 7), or 1:3 (lane 8) ratio. Immune-complex kinase assays and detection of the proteins were performed as described in Materials and Methods.

sion of 3pK with either kinase resulted in an enhanced activity of 3pK upon anisomycin treatment (Fig. 7A). A 2.5-fold increase was achieved by coexpression of SAPKβ relative to the control of cells transfected with 3pK alone. The same results were obtained after cotransfection of pEBG-3pK and HA-SAPKβ (data not shown). Further, coexpression of both, SEK1 or SAPKβ in unstimulated cells also resulted in an elevated 3pK activity. These data indicate that SAPK plays a role in stress-induced 3pK activation; however, the overall effect was not great. To examine which effect coexpression of p38RK might have, 293 cells were cotransfected with 3pK and p38RK in the presence or absence of the p38 inhibitor (Fig. 7B). p38RK cotransfection leads to only a threefold increase in 3pK, although the kinase clearly was demonstrated to be upstream 3pK in vivo. This finding was again supported by the effect of the p38 inhibitor SB203580 (Fig. 7B, lanes 5 and 6). The weak effect of SAPK and p38RK cotransfection might be due to the fact that stress-induced 3pK activation was already close to the maximum and could not be increased further. To test this suggestion, we generated a SEK1 mutant [SEK1(ST→EE)] that carries negative charged amino acids instead of serine 220 and threonine 224, which are known to be involved in SEK1 activation. This mutant is weakly active on JNK/SAPK in vivo, which allows examination of SAPK-mediated 3pK activation in the absence of extracellular stimuli. As seen with pEBG-SAPKβ, coexpression of 3pK with HA-SAPKβ results in a weak activation of 3pK without any stimulation (Fig. 7C). Coexpression of 3pK with increasing amounts of pEBG-SEK1(ST→EE) alone results in a marginal effect (Fig. 7C), which might be due to low levels of endogenous JNK/SAPK in 293 cells. If HA-SAPKβ is additionally coexpressed, 3pK activity is significantly enhanced, up to 4.5-fold (Fig. 7C), clearly indicating that 3pK activation is mediated by SAPK in this assay.

To rule out that the observed effects are the result of overexpression of the upstream kinases, we created kinase-dead mutants of SEK [pEBG-SEK(K→R)] and SAPK [(HA-SAPKβ(KK→RR)] by exchanging the conserved lysine(s) in the ATP-binding site of kinase subdomain II to arginine. Figure 7D shows that both mutants could efficiently interfere with anisomycin-induced 3pK activation, indicating that SEK and SAPK are part of the upstream activation pathway.

From these results, we conclude that 3pK is the first example of a protein kinase targeted by all three subgroups of the MAPK family (Fig. 8).

DISCUSSION

In this report, we have demonstrated that in contrast to RSK/MAPKAP-K1 and MAPKAP-K2, the protein kinase 3pK is activated by both stress inducers and mitogens in vivo in HL60 cells and transiently transfected 293 cells. Activation kinetics differ depending on the stimuli, in that a sustained 3pK activation is observed with stress inducers. Activation by mitogens is Raf dependent and is mediated by the Raf/MEK/ERK cascade. After stress stimulation of cells, 3pK activity is induced through pathways activating both p38RK and JNK/SAPK. 3pK is a first example of a kinase targeted by all three MAPK family members, ERK, p38RK, and JNK/SAPK. No protein kinase substrate was previously observed for JNK/SAPK. Thus, 3pK is a convergence point of mitogenic and stress signaling. With regard to the mechanism of 3pK activation, we have shown by mutational analysis that exchange of threonines 201 and 313 in potential MAPK phosphorylation sites to glutamic acid leads to constitutive activation of the kinase, suggesting that 3pK is activated by phosphorylation of these sites. Consistent with our earlier finding that 3pK and MAPKAP-K2 have partially overlapping substrate specificities (47), the small heat shock proteins become phosphorylated by 3pK in vitro at the same sites that are phosphorylated in vivo.

The fact that 3pK is activated by mitogens and stress inducers through parallel pathways suggests that the physiological role of the kinase is to regulate functions that are common to the three protein kinase cascades. The best-known cascade in terms of its physiological function is the classical Raf/MEK/ERK unit, which is essential for regulation of proliferation and differentiation processes (2, 39, 40). Further, activated Raf synergizes with Bcl-2 in preventing apoptosis (13, 54). The physiological consequences of stress kinase activation are less well understood. There is evidence that stress kinase cascades are involved in the biosynthesis of inflammatory cytokines (30, 44), in apoptotic processes (56), and in stress protection (29). It was proposed earlier that such a protective function might

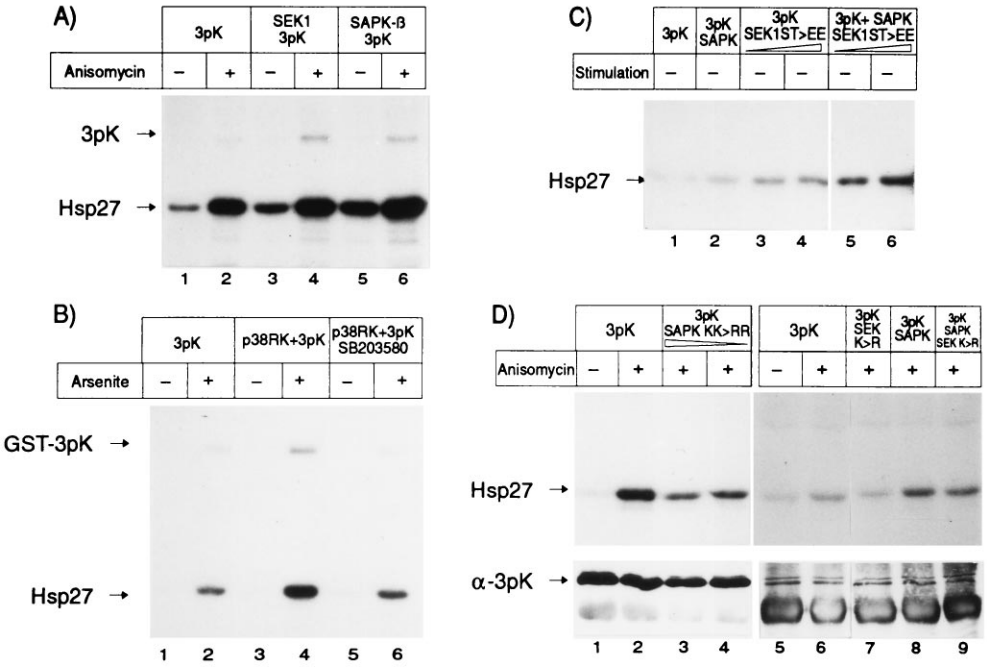

FIG. 7. Cotransfection of 293 cells with 3pK, SEK1, SAPKβ, p38RK, and different SEK and SAPKβ mutants. (A) 293 cells were transfected with RSV-3pK alone or in combination with pEBG-SAPKβ and pEBG-SEK1 as indicated and either left unstimulated (−) or stimulated with anisomycin for 30 min (+). 3pK was immunoprecipitated from cell lysates with the anti-3pK antibody and used in immune-complex kinase assays with Hsp27 as a substrate. (B) Cells were cotransfected with pEBG-3pK alone or in combination with pCDNA3-p38 and stimulated with arsenite in the presence or absence of SB203580 as indicated. (C) Cells were transfected with RSV-3pK alone or in combination with HA-SAPK and/or increasing amounts of the active mutant pEBG-SEK1(ST→EE) in a 1:2 (lanes 3 and 5) or 1:3 (lanes 4 and 6) ratio. (D) Cells were transfected with pEBG-3pK alone (3 μg) (lanes 1, 2, 5, and 6) or in combination with increasing amounts of HA-SAPKβ(KK→RR) (9 μg [lane 4] and 15 μg [lane 3]), pEBG-SEK(K→R) (15 μg) (lane 7), or HA-SAPKβ alone (1 μg) (lane 8) or in combination with pEBG-SEK(K→R) (9 μg) (lane 9). Cells were stimulated with anisomycin as indicated.

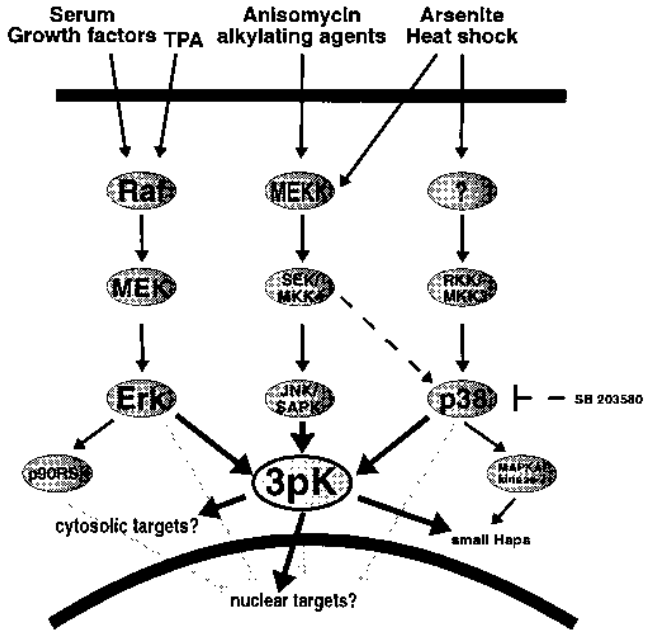

FIG. 8. 3pK in the framework of intracellular signaling. Position of the novel MAPKAP K 3pK in the network of signaling pathways defined by membrane shuttle kinases (Raf and MEKK), dual-specificity kinases (MEK, SEK/MKK4, and RKK/MKK3), and the nuclear shuttle kinases of the MAPK family (ERK, JNK/SAPK, and p38RK). Hsps, heat shock proteins.

also be necessary in processes induced after mitogenic stimulation (29). Thus, mitogenic cascades might share features with stress cascades, which may either be the protective response or the inactivation of checkpoint controls. 3pK may be a mediator of these common protective responses, as the kinase phosphorylates the small heat shock proteins.

While this report was in preparation, McLaughlin et al. (34) reported the identification of MAPKAP-K3, which is identical to 3pK, as a downstream substrate of p38RK, both in vitro and in vivo in stress-induced and transiently transfected COS and HeLa cells. These data differ from our findings in that the kinase was not activated by TPA in COS and HeLa cells, suggesting that activation might be cell type specific or dose dependent.

Mutational analyses have established threonines 201 and 313 as sites involved in activation of 3pK. While the latter site matches a consensus sequence PXTP for ERK phosphorylation, threonine 201 is located in a LXT/SP motif which is present at S-63 and S-73 in c-Jun and S-34 in p53, serine residues which are phosphorylated by JNKs/SAPKs (35, 36). This result again supports our finding that SAPK phosphorylates and activates 3pK, suggesting a physiological role for SAPK as an upstream kinase.

The essential difference of 3pK activation by mitogens versus stress stimulation is the sustained activation observed with stress-inducing agents. Maximal activity is observed after 30 to 60 min and is still at high levels after 4 h. This finding is consistent with a function of the kinase in stress protection, which requires a sustained activity. In contrast, downregulation of 3pK after mitogenic stimulation might be accomplished by a specific phosphatase activity, which is lacking under stress con-

ditions. We are currently analyzing the activation kinetics in different cell types in the hope of learning more about the physiological role of the enzyme.

The fact that 3pK phosphorylates the small heat shock proteins in vitro at the same sites that are phosphorylated in vivo might suggest that this is also an in vivo function of the kinase. In previous reports, MAPKAP-K2 was shown to be the major kinase for Hsp25 and Hsp27 (14, 49). However, these proteins are phosphorylated in response not only to stress inducers but also to mitogenic stimuli like serum and TPA (27). Since mitogenic stimuli will not activate MAPKAP-K2 (8, 44), it is still not known which kinase is responsible for mitogen induced in vivo phosphorylation of the small heat shock proteins. It was proposed earlier that stress-induced and mitogen-induced phosphorylation of Hsp27 is mediated by the same kinase (25, 60). 3pK is the most likely candidate since it is activated by both mitogens and stress inducers. However, it is unlikely that this is the only cellular function of 3pK. In fact, preliminary results point to a role of 3pK in the stress kinase-induced reporter gene transcription from an NF-κB-responsive promoter element and suggest a participation of 3pK in processes governing apoptosis of 32D cells (52a). In that respect, a potential nuclear localization signal located near the C-terminal end of 3pK (47) is of interest, since it suggests nuclear targets of the kinase. However, the functional significance of this motif remains to be evaluated.

The particular expression pattern of 3pK, with abundant presence in heart and skeletal muscle (47), further suggests a tissue-specific function. Since the Raf/MEK/ERK cascade was shown to be involved in the hypertrophic response of cardiomyocytes (5, 6, 51), 3pK may also be activated by hypertrophy-inducing agents. In this regard, it would be of interest to know whether the recently identified kinases MEK5 and ERK5 (21, 59), which are also highly expressed in the same tissues, are upstream activators of 3pK in myocytes. Experiments are under way to address these issues. In conclusion, our data identify 3pK as a novel convergence point of signaling downstream of MAPKs and may therefore be an important regulator of cellular functions common to all three cascades (Fig. 8).

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