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Metabolic labeling of mitogen-activated protein kinase kinase in A431 cells demonstrates phosphorylation on serine and threonine residues

(mitogen-activated protein kinase activator/MEK1/mitogen-activated protein kinase/extracellular signal-regulated kinase/epidermal growth factor)

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ABSTRACT **Mitogen-activated protein (MAP) kinase kinase is an enzyme that activates the growth factor-regulated MAP kinase *in vitro* by a mechanism that involves direct phosphorylation of MAP kinase on tyrosine and threonine residues. MAP kinase kinase is stimulated by growth factor treatment of cells and has been shown to be inactivated with protein phosphatases, suggesting that it is regulated by protein phosphorylation. Analysis of two epidermal growth factor-stimulated forms of MAP kinase kinase, purified from ^{32}P-labeled A431 cells, shows that the kinase is phosphorylated on serine and threonine residues and that treatment with protein phosphatases leads to serine dephosphorylation. Under conditions that lead to complete inactivation, only partial dephosphorylation of MAP kinase kinase is observed. Consistent with this finding, inactive forms of MAP kinase kinase, which separate from active forms during the course of purification, are also observed to be phosphorylated in intact cells.**

Several second messenger-independent kinases are activated in many cell types in response to a variety of mitogenic stimuli. Examples of these are (*i*) pp70^{rsk}, a ribosomal protein S6 kinase of 70 kDa (reviewed in ref. 1); (*ii*) pp90^{rsk}, a different ribosomal S6 protein kinase of 90 kDa, also referred to as S6 kinase II (1); (*iii*) extracellular signal-regulated kinases (ERKs), isoforms of 44 and 42 kDa (ERK1 and ERK2, respectively), also referred to as microtubule-associated protein 2 (MAP-2) kinase, mitogen-activated protein (MAP) kinase, or myelin basic protein (MBP) kinase (reviewed in ref. 2); (*iv*) pp54 MAP-2 kinase, a MAP-2 kinase of 54 kDa (3); and (*v*) Raf-1, a cellular form of the viral oncogene v-*raf* (74 kDa) (reviewed in ref. 4). In all of these examples, the protein kinases were reported to be inactivated by treatment with protein phosphatases, supporting the hypothesis that they are regulated by phosphorylation and are thus constituents in a protein kinase cascade analogous to the cascade of activation of cAMP-dependent protein kinase and phosphorylase kinase involved in the regulation of glycogen phosphorylase (5).

The first evidence for a growth factor-stimulated protein kinase cascade was demonstrated by activation of pp90^{rsk} by ERK1 or ERK2 (6–8). Rapid progress in characterizing this pathway has led to the findings that (*i*) phosphorylation of MAP kinases on threonine and tyrosine is required for activation (9–11); (*ii*) the sites of phosphorylation on ERK2 are Thr-183 and Tyr-185, both located on a single tryptic fragment in subdomain VIII of the conserved catalytic domain sequence (12); and (*iii*) ERK1 and ERK2 autophosphorylate on threonine and tyrosine residues, suggesting that they belong to a category of "dual specificity" protein kinases, which phosphorylate both tyrosine and serine/threonine residues (13–16).

A MAP kinase kinase (MKK) has been identified that is capable of activating MAP kinase *in vitro* (reviewed in ref. 17). Stimulation of this enzyme has been demonstrated in epidermal growth factor or phorbol 12-myristate 13-acetate (PMA)-stimulated Swiss 3T3 cells, nerve growth factor or bradykinin-stimulated PC12 cells, PMA-stimulated lymphocytes, *Xenopus laevis* oocytes induced to undergo maturation, and insulin-stimulated rabbit muscle (18–25). Upon activation, threonine and tyrosine residues on MAP kinase are phosphorylated, and analyses of phosphorylated tryptic peptides and of mutant forms of MAP kinase suggest that this occurs on the relevant phosphorylation sites *in vivo* (19, 22, 25). MKK has been purified from *Xenopus*, human, rabbit, and mouse cell extracts (23, 26–28), and cDNA sequences of the mouse, human, rabbit, rat, and *Xenopus* enzymes have been reported (29–33). All show a close relationship to the yeast signal transduction protein kinases, STE7 from *Saccharomyces cerevisiae*, and byr1 from *Schizosaccharomyces pombe*, which are part of related kinase cascades that function in yeast mating (reviewed in refs. 34 and 35).

MKK can be inactivated by treatment with protein phosphatases, suggesting that it is regulated by phosphorylation (20, 23, 27, 36). MKK has been shown to autophosphorylate on tyrosine as well as serine and threonine residues *in vitro* (26, 28, 37). Regulation of MKK has been suggested to occur through serine/threonine phosphorylation, since it is inactivated by the serine/threonine-specific protein phosphatase 2A (20), although this phosphatase can also dephosphorylate phosphotyrosine (38). Nevertheless, metabolic labeling of MKK in *X. laevis* oocytes has demonstrated the presence of phosphoserine and phosphothreonine on the enzyme (37, 39). In these studies, the phosphothreonine was sensitive to dephosphorylation by phosphatase 2A (37).

In this study, we have purified MKK from ^{32}P-labeled A431 cells and demonstrated that it is phosphorylated on serine and threonine residues. Tyrosine phosphorylation was not observed. Under conditions that led to complete loss of activity, treatment of MKK with protein phosphatases resulted in loss of only ≈30% of the ^{32}P radioisotope. The results suggest that this enzyme is phosphorylated on serine/threonine residues, some (but not necessarily all) of which contribute to activity.

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Abbreviations: MAP, mitogen-activated protein; MKK, MAP kinase kinase; MBP, myelin basic protein; ERK, extracellular signal-regulated kinase; PP2A, protein phosphatase 2A; PP1, protein phosphatase 1; TX100, Triton X-100; G protein, guanine nucleotide-binding protein.

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MATERIALS AND METHODS

Preparation and Treatment of Cells. Human A431 cells were grown on 30 150-mm plastic Petri dishes in 25 ml of medium (10% fetal bovine serum/Dulbecco–Vogt-modified Eagle's medium). After the cells reached a confluent monolayer, the medium in four dishes was replaced with 10 ml of medium containing low phosphate (fetal serum was dialyzed against 0.15 M NaCl and Dulbecco–Vogt-modified Eagle's medium was made with 0.1 mM sodium phosphate). To each dish, 2.5 mCi of $^{32}P_i$ (10 mCi/ml; 1 Ci = 37 GBq; NEN) was then added. The remaining dishes were refed with 10 ml of medium. The cells were incubated 12–15 hr at 37°C. Cells were then treated with epidermal growth factor (100 ng/ml; 5 min), washed twice with 5 ml of cold phosphate-buffered saline, and washed once with 5 ml of homogenization buffer (50 mM β-glycerophosphate, pH 7.4/1.5 mM EGTA/0.1 mM sodium orthovanadate/1 mM dithiothreitol/10 μg of leupeptin per ml/10 μg of aprotinin per ml/2 μg of pepstatin A per ml/1 mM benzamidine), scraped, and disrupted by sonication (50 W; 15 s). Extracts were then centrifuged at 100,000 × *g* and the supernatants from labeled cells and nonlabeled cells were pooled.

Assay and Purification of MKK. MKK was assayed by its ability to stimulate MAP kinase contained in "B3," which is a pool of inactive ERK1 and ERK2 derived from quiescent Swiss 3T3 cells (18). The activity of ERK1 and ERK2 was measured by using MBP as substrate (18). Preparation of B3 and this coupled assay for MKK are described (18, 19, 26).

MKK was purified from ^{32}P-labeled A431 cells. Unless noted, protocols and reagents were as described (26). Cytosolic extracts from 30 150-mm dishes (≈20 ml) were diluted 1:1 with 25 mM Hepes, pH 7.4/1 mM dithiothreitol and applied to a Q Sepharose column (20 ml; 1 ml/min) equilibrated in buffer C [25 mM β-glycerophosphate, pH 7.4/12.5 mM Hepes/0.75 mM EGTA/0.05 mM sodium orthovanadate/1 mM dithiothreitol/5% (vol/vol) glycerol], and eluted with a linear gradient of NaCl (0–0.4 M in 200 ml of buffer C; 1.5-ml fractions). Two overlapping peaks of MKK eluted between 0.12 and 0.2 M NaCl, resolving from MAP kinase, which eluted at 0.3 M NaCl. Fractions containing both peaks of MKK were pooled (40 ml), diluted 1:3 with buffer C/0.01% Triton X-100 (TX100), and applied to a heparin/agarose column (10 ml; 1 ml/min), which was developed with 0–0.3 M NaCl in buffer C/0.01% TX100 (120 ml; 1.5-ml fractions). MKK eluted between 0.1 and 0.15 M NaCl. Fractions containing activity were pooled (20 ml), applied to a hydroxylapatite/CF11 cellulose column (4 ml; 1 ml/min), and eluted with 0–0.3 M potassium phosphate in buffer C/0.01% TX100 (40 ml; 1-ml fractions). MKK eluted between 0.1 and 0.18 M phosphate. Fractions containing activity were pooled (11 ml) and dialyzed with simultaneous concentration against buffer C/0.01% TX100. The sample (3 ml) was applied to an ATP agarose column (0.2 ml; 0.25 ml/min) and the breakthrough was collected and further concentrated. The sample (0.25 ml) was then applied to a Superose 12 HR 10/30 column (Pharmacia; 25 ml; 0.3 ml/min) equilibrated in buffer D (40 mM Hepes, pH 7.4/2 mM EDTA/2 mM dithiothreitol/5% glycerol). MKK eluted from this column as a single peak with a molecular mass of 55–60 kDa, compared to molecular size standards. Fractions containing activity were pooled (2 ml), applied to a Mono S HR 5/5 column (Pharmacia; 1 ml; 0.5 ml/min), and eluted with 0–0.3 M NaCl in buffer D (20 ml; 0.5-ml fractions were collected in tubes preloaded with 5 μl of 1% TX100). MKK eluted at 0.14 M NaCl. Fractions containing activity were pooled, diluted 1:1 with buffer D/0.01% TX100, applied to a Mono Q HR 5/5 column (Pharmacia; 1 ml; 0.5 ml/min), and eluted with a gradient of 0–0.3 M NaCl in buffer D (20 ml; 0.5-ml fractions were collected in tubes preloaded with 5 μl of 1% TX100). Purification was carried out six times with similar results.

Western Blotting. Rabbit antiserum Ab2167 (31) was used in Western blots (1:1000 dilution) that were developed with alkaline phosphatase-coupled goat anti-rabbit antibodies and 4-nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate (Promega).

Inactivation of MKK. Mono S fractions containing MKK activity were pooled and incubated with protein phosphatase 1 (PP1) (500 units/ml; 1 unit = 1 nmol/min vs. phosphorylase a) or bovine heart protein phosphatase 2A (PP2A) (50 units/ml) at 30°C. Catalytic subunit from rabbit skeletal muscle PP1 was purified as described (40). Catalytic subunit of bovine heart PP2A was a gift of Timothy Haystead (University of Virginia). Controls were performed by including microcystin (1 μM; LC Services, Woburn, MA) in the PP1 incubations or okadaic acid (1 μM; LC Services) in the PP2A incubations. At various times, aliquots (1.5 μl) were removed to a mixture of B3 (11 μl) and assay buffer containing Mg[γ-^{32}P]ATP (30 mM $MgCl_2$/0.3 mM ATP; 2000 cpm/pmol) and 3 μM microcystin or okadaic acid (8.33 μl). Other components in the assay buffer have been described (18, 26). After 15 min of preincubation, MBP (4.2 μl; 2 mg/ml) was added and the reaction was carried out for an additional 20 min to measure MKK activity by the stimulation of ERK1 and ERK2. Activity measurements were corrected for endogenous MBP kinase activity in B3. Alternatively, Laemmli sample buffer was added to the reaction mixture and samples were separated by SDS/PAGE (10% acrylamide) to assay MKK activity by direct phosphorylation of ERK1 and ERK2.

Phosphoamino Acid and Phosphorimager Analyses. Samples were resolved by SDS/PAGE (10% acrylamide) and transferred electrophoretically to Immobilon-P membranes (Millipore). After visualization by autoradiography, membrane slices containing radiolabeled proteins were excised and hydrolyzed in constant boiling HCl (41) prior to two-dimensional phosphoamino acid analysis (42). Phosphorimager analysis was performed by Margot Gibson (Markey Phosphorimager Facility, University of Washington) on a Molecular Dynamics phosphorimager (model 4005) after a 16-hr exposure to the phosphorescent screen.

RESULTS

In our previous studies, two forms of MKK (MKK-1 and -2) were found in Swiss 3T3 and A431 cells and were purified to homogeneity from A431 cells (18, 26). These eluted first and second on anion-exchange chromatography and corresponded to 46- and 45-kDa proteins by SDS/PAGE (26). Chromatographic behavior of these enzymes was nearly identical in all other steps. In the current study, MKK-1 and -2 partially resolved on the first step (Q Sepharose) but were pooled together and copurified until the final step (Mono Q). Elution of MKK activity from the last two steps (Mono S and Mono Q) are shown (Fig. 1 *A* and *B*). MKK eluted as a single broad peak on Mono S (Fig. 1*A*), which resolved into two peaks (MKK-1 and -2) on Mono Q, eluting at 0.1 and 0.18 M NaCl, respectively (Fig. 1*B*). The activity in the second peak was consistently lower than that in the first peak.

Silver staining of fractions from Mono Q (Fig. 1*D*) showed the presence of two proteins that had been previously identified as MKK-1 and -2 (peaks of activity denoted by arrows in Fig. 1 *B* and *D*). On the SDS/PAGE system used, both proteins migrated as 45-kDa polypeptides. These were recognized by Western blotting with Ab2167 sera (data not shown). Both 45-kDa polypeptides were phosphorylated and represented the only ^{32}P-labeled proteins found in these fractions (Fig. 1*F*).

Silver staining of fractions from the preceding column (Mono S; Fig. 1*C* and 2*A*) showed that the peak of the 45-kDa

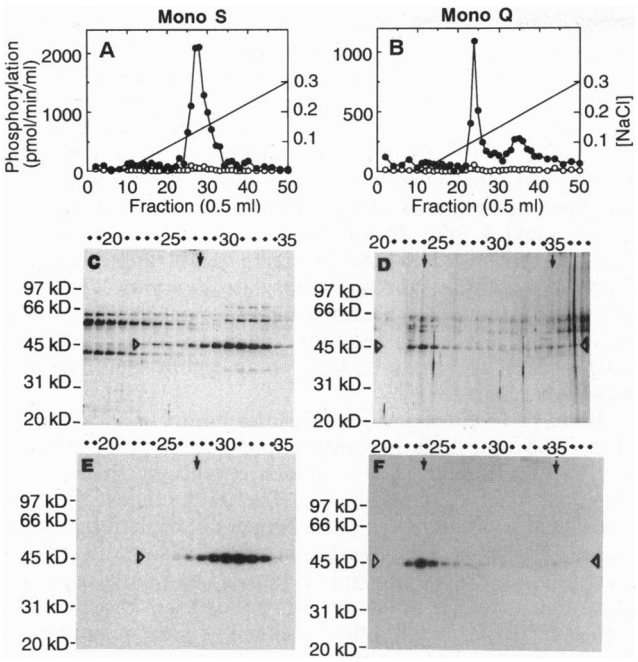

FIG. 1. Chromatography of MKK. Elution of MKK from Mono S (*A*) and Mono Q (*B*) as described. Fractions were diluted 1:20 with column buffer and assayed for activation of MAP kinase after preincubation with B3 (●) or buffer (○). Elution gradient of NaCl is indicated by straight line. (*C* and *D*) Silver staining of fractions (60 μl) from Mono S (*C*) and Mono Q (*D*) columns after separation by SDS/PAGE. (*E* and *F*) Autoradiography of gels in *C* and *D*, respectively. Vertical arrows denote MKK activity peaks. Arrowheads denote 45-kDa MKK polypeptides.

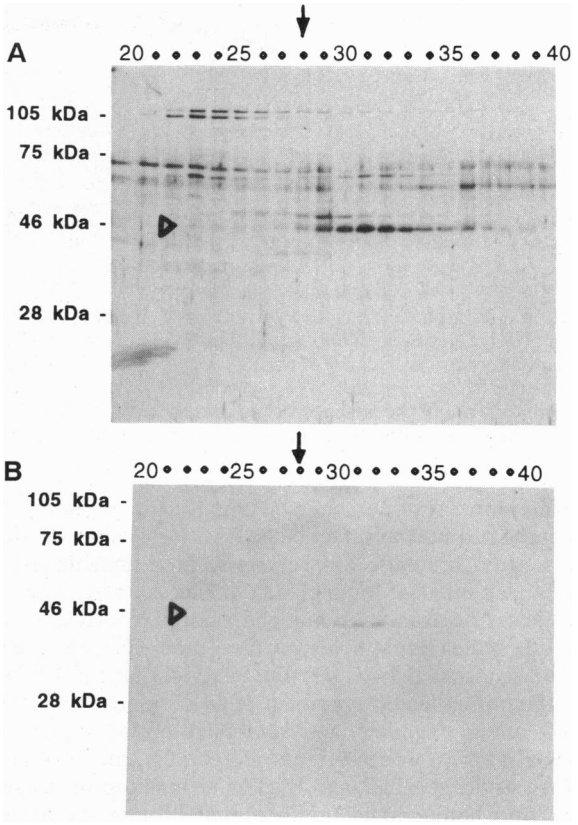

FIG. 2. Immunoreactivity of MKK. (*A*) Silver staining of fractions (60 μl) from Mono S. (*B*) Western blotting of fractions (20 μl) from Mono S using Ab2167. Vertical arrows indicate MKK activity peak. Arrowheads indicate 45-kDa MKK polypeptide.

polypeptide consistently trailed three or four fractions behind the peak of MKK activity. This was recognized by Ab2167 (Fig. 2*B*) and therefore was immunologically crossreactive with MKK. The data suggest that the major amount of MKK is an inactive form, resolving from the active form on Mono S. Autoradiographic exposure of the silver-stained gel (Fig. 1*C*) showed that both active and inactive forms of MKK are phosphorylated (Fig. 1*E*).

Phosphoamino acid analysis indicated that MKK-1 and -2, eluting from Mono Q, are both phosphorylated on serine and threonine, but not tyrosine, residues (Fig. 3). Analysis of proteins eluting from Mono S also showed the presence of phosphoserine and phosphothreonine but not phosphotyrosine in both the active (Mono S fractions 25–30, as in Fig. 1) and inactive (Mono S fractions 31–33, as in Fig. 1) forms of MKK (data not shown). The relative amounts of phosphoserine and phosphothreonine were the same for the inactive and active enzyme (data not shown).

MKK from the Mono S column was inactivated upon treatment with protein phosphatases, consistent with the behavior of the enzyme from rat and *X. laevis* sources (20, 23). PP1, a serine/threonine phosphatase with no reported tyrosine phosphatase activity, was able to completely inactivate MKK (Fig. 4*A*), supporting a mechanism of inactivation involving serine/threonine dephosphorylation. PP2A, a serine/threonine phosphatase, which can also dephosphorylate phosphotyrosine under some conditions (40), was also able to inactivate MKK (Fig. 4*B*). Inactivation by several protein tyrosine phosphatases (CD45, TC20, rPTPa, or PTP1C) was not observed (data not shown). In these experiments, inactivation of MKK was measured as a loss of ERK1 and ERK2 activation. Inactivation could also be demonstrated as a complete loss of ERK1 and ERK2 phosphorylation (data not shown), indicating that both tyrosine and threonine phosphorylation of ERK1 and ERK2 were inhibited.

The effect of PP2A on MKK phosphorylation was measured in parallel reactions (Fig. 5 *A* and *B*). Under conditions that led to complete inactivation, only ≈30% dephosphorylation of MKK was observed (Fig. 5*B*), which correlated predominantly with loss of phosphoserine (Fig. 5*C*). Similar results were obtained after PP1 inactivation (data not shown). The data indicate that MKK activity is regulated by serine and perhaps threonine phosphorylation but that complete dephosphorylation is not required for inactivation.

DISCUSSION

Our major finding in this study is that MKK, a growth factor-regulated kinase with specificity for tyrosine and ser-

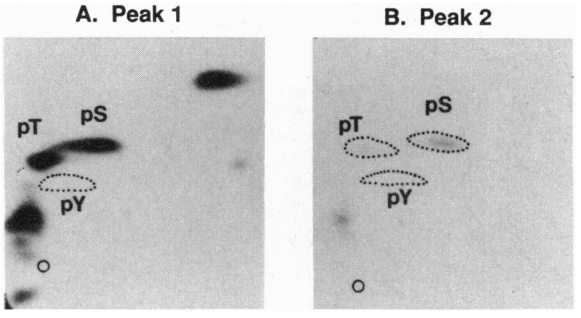

FIG. 3. Phosphoamino acid analysis of MKK-1 and -2. Phosphoproteins in fractions 26–28 (*A*) or 34–36 (*B*) from Mono Q were pooled and purified by SDS/PAGE before phosphoamino acid analysis as described. Phosphoserine, phosphothreonine, and phosphotyrosine are denoted by pS, pT, and pY, respectively.

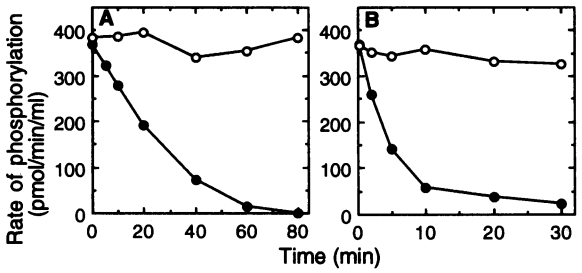

FIG. 4. Phosphatase inactivation of MKK by PP1 and PP2A. Active MKK, eluting in Mono S fractions 25–30 (as in Fig. 1), was incubated for various lengths of time with PP1 (500 units/ml) (*A*) in the absence (●) or presence (○) of microcystin (1 μM), or with PP2A (50 units/ml) (*B*) in the absence (●) or presence (○) of okadaic acid (1 μM). MKK activity was then measured by activation of B3 as described.

ine/threonine residues, is phosphorylated on serine and threonine residues in intact cells. This implies the existence of a serine/threonine kinase(s) activator immediately upstream. A candidate for such an enzyme is Raf-1, which has been shown to activate and phosphorylate MKK *in vitro* (43–45). Other protein serine/threonine kinases, such as protein kinase C (18, 21, 46, 47), c-src (48), and $p34^{cdc2}$ (23), have been implicated upstream of MAP kinase and MKK, based on biochemical and pharmacological studies performed in various cell systems. Potentially, mammalian homologs of yeast STE7 and byr1 may function upstream of MKK in guanine nucleotide-binding protein (G protein)-coupled signaling pathways as well. *In vitro*, MKK is a substrate for $p34^{cdc2}$ and MAP kinase, although direct activation by these enzymes has not been demonstrated (N.G.A., unpublished data). Recent *in vitro* studies have also suggested that the serine/threonine kinase-encoding protooncogene c-*mos* may directly phosphorylate and activate MKK, and microinjection studies have shown that c-*mos* functions upstream of MKK during maturation of intact *X. laevis* oocytes (49). In addition, a protein factor of high molecular mass (>440 kDa; by sizing gel filtration) has been reported to activate and phosphorylate *Xenopus* MKK (39). Thus, MKK may be a common target for several kinases, in some cases present in specific cell types, that function to couple the MAP kinase cascade to divergent signaling pathways upstream.

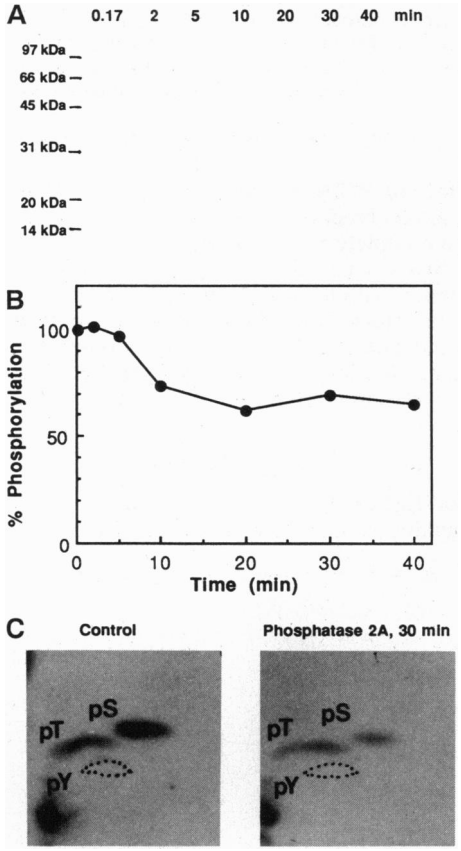

FIG. 5. Dephosphorylation of MKK by PP2A. (*A*) Autoradiograph of active ^{32}P-labeled MKK (eluted from Mono S) during incubation with PP2A (50 units/ml). (*B*) ^{32}P in MKK was quantified with a phosphorimager and plotted on a relative scale. (*C*) MKK was untreated (*Left*) or treated with PP2A (50 units/ml; 30 min) (*Right*) before phosphoamino acid analysis. Abbreviations are as in Fig. 3.

We have found that a significant amount of serine phosphate, and almost all threonine phosphate, remains resistant to PP1 or PP2A treatment, under conditions that lead to complete inactivation of MKK. The data suggest the existence of at least two sites of serine phosphorylation and one site of threonine phosphorylation, if we assume that phosphatase completely dephosphorylates a key regulatory phosphoamino acid. At least one phosphoserine is critical for regulating activity. The other sites may reflect residues that are also essential for activity but that are not recognized by PP1 or PP2A. Alternatively, they may regulate functions of the enzyme other than activity. Significantly, metabolic labeling studies performed on MKK from *X. laevis* showed the presence of phosphothreonine and low amounts of phosphoserine (37, 39). The phosphothreonine could be removed by protein phosphatase treatment (37), but it is unknown whether the phosphoserine is susceptible to dephosphorylation.

Several laboratories, including our own, have demonstrated autophosphorylation of MKK on serine, threonine, and tyrosine residues *in vitro* (26, 28, 38). Our results indicate that the enzyme does not require tyrosine phosphorylation for activity, consistent with the inability to inactivate MKK with several protein tyrosine phosphatases (data not shown; see ref. 20). However, the data do not exclude the possibility that tyrosine phosphorylation occurs *in vivo*, followed by rapid tyrosine dephosphorylation. The contribution of serine/threonine autophosphorylation to activation is unknown; however, we have been unable to reactivate phosphatase-treated MKK upon autophosphorylation, supporting the prevailing model that a separate kinase is involved.

Our results also demonstrate the existence of three forms of MKK that resolve on anion- and cation-exchange chromatography. All three forms are crossreactive with a polyclonal antibody raised against an N-terminal peptide epitope, suggesting that they are at least partially related by amino acid sequence. Two forms, described previously as MKK-1 and -2, respectively, eluting first and second on anion-exchange chromatography, both show activity toward MAP kinase. A third, inactive form of MKK partially resolves from active MKK-1 and -2 by Mono S chromatography. The inactive enzyme appears to be the major form of MKK protein present in these preparations, suggesting that only a fraction of the enzyme may be activated upon cell stimulation, under conditions optimized with respect to time and epidermal growth factor concentration. Similar results have been found with MKK purified from rabbit skeletal muscle (L.M.G. and E.G.K., unpublished data). Both active and inactive forms are phosphorylated on serine and threonine residues and are indistinguishable by phosphoamino acid analysis. Their relationship to each other may reflect subtle changes in their state of phosphorylation. Alternatively, they may differ by other posttranslational modifications or by amino acid sequence. It is interesting to note that, although cDNA clones of human, rabbit, rat, and mouse MKK predict

proteins with ≈99% amino acid sequence identity to each other (29–32), some evidence for heterogeneity has been found in the identification of a cDNA encoding an alternatively spliced form of human MKK (31).

Our results support and extend the findings of others who have demonstrated the activation and phosphorylation of MKK by serine/threonine-specific kinases *in vitro* (43–45, 49). In intact cells, MKK is activated through multiple signaling pathways involving tyrosine kinase receptors, G-protein-coupled receptors, and progesterone receptors. The *in vitro* activation of MKK by Raf-1, a serine/threonine kinase that is activated by tyrosine kinase receptor-linked signals but not by signals that stimulate G-protein-coupled receptors, suggests that MKK may be a point of integration between multiple signaling pathways. It will be interesting to see whether the phosphorylation of MKK on multiple sites provides a means by which information from different extracellular signals can converge on the regulation of the MAP kinase cascade.

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