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Growth Factor-Induced Activation of a Kinase Activity Which Causes Regulatory Phosphorylation of p42/Microtubule-Associated Protein Kinase

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p42/microtubule-associated protein kinase ($p42^{mapk}$) is activated by tyrosine and threonine phosphorylation, and its regulatory phosphorylation is likely to be important in signalling pathways involved in growth control, secretion, and differentiation. Here we show that treatment of quiescent 3T3 cells with diverse agonists results in the appearance of an activity capable of causing the in vitro phosphorylation of $p42^{mapk}$ on the regulatory tyrosine and to a lesser extent on the regulatory threonine, resulting in enzymatic activation of the $p42^{mapk}$. This $p42^{mapk}$-activating activity is capable of phosphorylating a kinase-defective $p42^{mapk}$ mutant, thus confirming its activity as a kinase.

p42/microtubule-associated protein (MAP) kinase ($p42^{mapk}$) is a serine/threonine protein kinase which rapidly becomes phosphorylated on threonine and tyrosine (42) in quiescent cells stimulated with various agonists, resulting in enzymatic activation. Both the threonine and tyrosine phosphorylations are necessary for full enzymatic activation (7, 27, 44), and the phosphorylation sites have been identified as T-183 and Y-185 (35).

$p42^{mapk}$ is the paradigmatic member of an enzyme family which is activated by agonist stimulation in various cell types and species (for a recent review, see reference 12). Members of this family have also been termed MAP-2 kinases (23, 27, 40–42), myelin basic protein (MBP) kinases (2, 4, 44) or extracellularly regulated kinases (ERKs) (9, 10). The $p42^{mapk}$ studied here corresponds to the MAP-2 kinase originally described by Ray and Sturgill (40), the MBP kinase 1 of Krebs et al. (2, 4, 44), the ERK2 of Boulton et al. (9, 10), and the tyrosine phosphorylated protein pp42 described earlier by Cooper and Hunter (13, 24) and by our (8, 42) and other laboratories (17, 26).

Several candidate substrates for $p42^{mapk}$ and its family members have been identified, including MAP-2 (40), *rsk* ribosomal S6 kinase (11, 47), *raf* protein kinase (6), epidermal growth factor (EGF) receptor (35), and *jun* transcription factor (39). Thus, $p42^{mapk}$ and its relatives are likely to play a significant role in signalling cascades, especially those involving other kinases.

Phosphorylation and activation of $p42^{mapk}$ and/or members of this enzyme family are associated with $G_0 \rightarrow G_1$ transitions (29, 38, 43), M phase (19, 20, 38), neuronal differentiation (21, 32, 49), lymphocyte activation (33), and secretion (15). Stimulatory agonists include the following: (i) agents which activate tyrosine kinase receptors, such as insulin (40–42), EGF (3, 21, 23, 43), platelet-derived growth factor (PDGF) (25, 30), nerve growth factor (10, 18, 21, 49), and fibroblast growth factor (23, 29, 49); (ii) protein kinase C agonists, such as the tumor promoter tetradecanoyl phorbol acetate (TPA) (1, 8, 17, 30); and (iii) agonists for G proteins, such as fluoroaluminate (5, 34) and thrombin (29). Because of the diversity of cell types and agonists which are associated with activation of this enzyme, it has been proposed that $p42^{mapk}$ plays an important role in integrating diverse regulatory signals. Understanding how this enzyme is regulated is thus important for elucidating the signalling pathways used by growth factors and numerous other stimulatory agents.

Any one or a combination of three general mechanisms could be responsible for the regulatory phosphorylation of $p42^{mapk}$: (i) a kinase cascade, in which an upstream kinase is regulated by agonists and phosphorylates $p42^{mapk}$, much as $p42^{mapk}$ phosphorylates *rsk* kinase (47); (ii) an intramolecular autokinase reaction, in which a nonkinase cellular factor regulates the ability of $p42^{mapk}$ to phosphorylate itself; (iii) an intermolecular autokinase reaction, in which $p42^{mapk}$ molecules are activated allosterically and then engage in mutual transphosphorylations, as occurs with many tyrosine kinase receptors.

Previous work has demonstrated that $p42^{mapk}$ is capable of slow intramolecular autophosphorylation (14, 46, 51) on tyrosine 185 (51), the site of in vivo regulatory phosphorylation on tyrosine. This reaction is accompanied by partial enzymatic activation. Because $p42^{mapk}$ is capable of this basal, regulatory autophosphorylation, we and others have speculated that the autokinase activity might be exploited in the regulatory phosphorylation which accompanies agonist stimulation of quiescent cells (46, 51). That is, agonist stimulation might activate a regulatory factor capable of enhancing the rate of tyrosine autophosphorylation, providing a novel mechanism for enzymatic activation by phosphorylation.

In this report, we demonstrate an activity in lysates from agonist-activated Swiss 3T3 cells capable of phosphorylating a kinase-defective $p42^{mapk}$ substrate on the sites of regulatory phosphorylation, threonine 183 and tyrosine 185. This phosphorylation leads to enzymatic activation of the $p42^{mapk}$ substrate. Thus a kinase cascade mechanism appears to be

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the major mechanism for agonist-stimulated phosphorylation and activation of p42^{mapk} measured in this system.

MATERIALS AND METHODS

Materials. Human recombinant EGF and human recombinant PDGF (BB form) were from Upstate Biotechnology, Inc., Lake Placid, N.Y. TPA and MBP were from Sigma Chemical Co., St. Louis, Mo. Phenyl-Sepharose CL-4B resin, the G25 prepacked columns, and the fast-performance liquid chromatography (FPLC) columns (MonoQ and MonoS) were from Pharmacia-LKB, Piscataway, N.J. [γ-^{32}P]ATP (6,000 Ci/mmol) was from NEN-DuPont Research Products, Wilmington, Del. P81 phosphocellulose paper was from Whatman, Clifton, N.J. Microconcentrators (Centricon 10) were from Amicon, Beverly, Mass. The colorimetric protein assay used in this study was from Bio-Rad, Richmond, Calif.

Specific phosphotyrosine antibodies and p42^{mapk}/p44^{mapk}-specific antibodies (raised against the carboxy-terminal 10 amino acids of the predicted ERK1 sequence) were generated in this laboratory as described previously (28, 43). Purified phosphatase 2A and okadaic acid were kindly provided by Timothy Haystead, Pharmacology Department, University of Virginia, Charlottesville, Va.

Mutagenesis, expression, and protein purification. All the indicated point mutations were generated by using the Altered Sites System (Promega, Madison, Wis.) and were confirmed by DNA sequencing. The recombinant p42^{mapk} proteins, wild type and mutant, were expressed in *Escherichia coli* by induction with isopropyl-β-D-thiogalactopyranoside as described previously (51). Bacteria were then sonicated in a buffer solution containing 25 mM Tris (pH 7.4), 25 mM NaCl, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 2 mM EDTA. Lysates were clarified by centrifugation at 10,000 × *g* for 5 min before adsorption to phenyl-Sepharose. The resin was washed with the same buffer containing 10% and then 25% ethylene glycol before elution of the recombinant p42^{mapk} with buffer containing 60% ethylene glycol. Alternately, the wild-type recombinant and kinase-defective proteins were more extensively purified through phenyl-Superose (51); the nonphosphorylated form of the wild-type recombinant was used. The concentrations of the recombinant proteins were determined both by immunoblotting with the anti-MAP kinase antibody and by colorimetric protein assay, so that the same amounts of wild-type and mutant proteins could be utilized for the kinase reactions. Immunoblotting was done as previously described (30).

Cell culture. Confluent cultures of Swiss 3T3 fibroblasts were rendered quiescent by an overnight serum starvation in Dulbecco's modified Eagle medium before agonist stimulation. Cells were then lysed by Dounce homogenization in ice-cold hypotonic buffer containing 25 mM Tris (pH 7.4), 25 mM NaCl, 1 mM dithiothreitol, 40 mM *p*-nitrophenyl phosphate, 1 mM $NaVO_4$, 1 mM phenylmethylsulfonyl fluoride, 10 μg of aprotinin per ml, 10 μg of leupeptin per ml, and 0.5 mM pepstatin A, as described previously (30, 40, 41). Lysates were clarified by centrifugation at 20,000 × *g* for 10 min and stabilized in buffer containing 10% ethylene glycol before freezing or were used immediately for kinase reactions or further purification.

Kinase assays. In vitro phosphorylation reactions were carried out at 30°C for 20 min in kinase buffer (25 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid] [pH 7.4] 10 mM magnesium acetate, 1 mM dithiothreitol, and 50 μM [γ-^{32}P]ATP [5,000 cpm/pmol]) with unpurified or FPLC-purified cytosol as the activator and recombinant p42^{mapk} proteins as the substrate. Reactions were terminated by adding hot sample buffer (1% sodium dodecyl sulfate [SDS], 50 mM dithiothreitol, 10% glycerol, 0.05 M Tris, pH 6.8) and analyzed by SDS–polyacrylamide gel electrophoresis (PAGE). The 20-min time point shown in this communication is in the linear range of this in vitro phosphorylation whether the cytosol used was FPLC purified or not (our unpublished observations).

The MBP kinase activity of FPLC-purified material was assayed in the presence of the wild-type p42^{mapk} (0.3 μg per assay) as the activatable substrate and conducted at 30°C in the same kinase buffer except that 0.5 mg of MBP per ml and 50 μM [γ-^{32}P]ATP (1,000 cpm/pmol) was used. The levels of ^{32}P-labeled MBP were assessed by 15% SDS–PAGE or by spotting the reaction mixture onto P81 phosphocellulose paper as described previously (16).

FPLC purification. Clarified cytosol extracts from 3T3 cells were fractionated through FPLC by using an anion-exchange MonoQ column (5 by 0.5 cm) and then a cation-exchange MonoS column (5 by 0.5 cm). MonoQ fractionation was performed in the same cell lysis buffer as for cytosol preparation with a 20-ml linear salt gradient to 250 mM NaCl. MonoQ fractions were then analyzed, first for their ability to phosphorylate in vitro the kinase-defective K52R mutant protein (the MAP kinase kinase [MAPKK] activity) and second for their capacity to enzymatically activate the wild-type recombinant nonphosphorylated form of the p42^{mapk} protein, measured by appearance of MBP kinase activity. (MBP serves in these assays as an in vitro substrate for the enzymatically activated MAP kinase.)

Phosphoamino acid analysis. Phosphorylated bands at 42 kDa were excised from SDS-polyacrylamide gels, eluted in 25 mM *N*-ethylmorpholine (pH 7.7), boiled 5 min with 0.1% SDS–5% β-mercaptoethanol, and extracted for 3 h. Supernatants were precipitated with 50 μg of poly(Glu-Tyr) carrier plus 20% trichloroacetic acid on ice for 4 h. The pellets were then washed and lyophilized first with cold ethanol and then with water. Finally, the samples were dissolved in 5 μl of pyridine acetate (pH 3.5) (water-acetic acid-pyridine, 945:50:5) containing the P-Ser, P-Thr, and P-Tyr markers, subjected to electrophoresis on thin-layer cellulose at 1 kV for 1 h, air dried, and autoradiographed. Typically, a protein of 43 to 44 kDa endogenously present in the lysates underwent phosphorylation and was difficult to separate from the p42^{mapk} band; this was responsible for the phosphoserine signal.

RESULTS

Construction and expression of p42^{mapk} mutant kinases. To study the mechanisms of p42^{mapk} activation, we constructed by site-directed mutagenesis K52R, a mutant protein defective in kinase activity, by converting lysine 52 in the ATP binding site (22) to arginine. We also constructed mutants in which the amino acids involved in regulatory phosphorylation (36), threonine 183 and tyrosine 185, were changed to alanine or phenylalanine, respectively, or were changed to glutamate to mimic the negative charge of phosphate. In addition to the singly mutated forms, the double mutants T183A/Y185F and T183E/Y185E were constructed.

Full-length, wild-type p42^{mapk}, and each of the mutants were expressed in *E. coli*, using the expression system previously described (51). Lysates of bacteria expressing these proteins were subjected to SDS-PAGE and immuno-

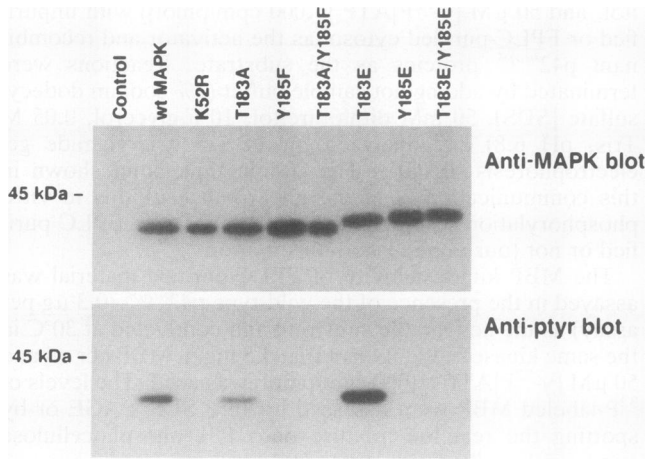

FIG. 1. Expression and tyrosine phosphorylation of recombinant $p42^{mapk}$ in bacteria. The bacterial lysates were prepared as described in Materials and Methods, then separated by SDS-PAGE, transferred to nitrocellulose, and blotted either with the MAP kinase (MAPK) antibody or with the antiphosphotyrosine (ptyr) antibody. wt, wild type.

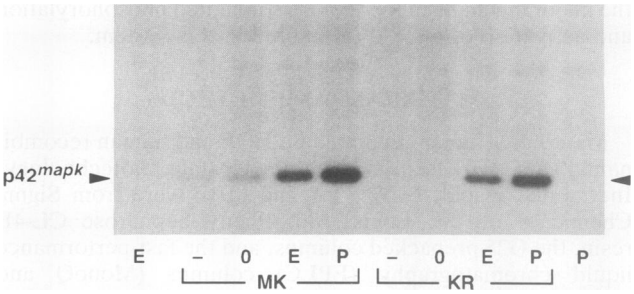

FIG. 2. In vitro phosphorylation of wild-type (MK) and kinase-defective (KR) $p42^{mapk}$ by cytosolic extracts of 3T3 cells activated with EGF or PDGF. Quiescent cells were stimulated for 5 min with 300 ng of EGF (E) per ml or 20 ng of PDGF (P) per ml or were left quiescent (0) before lysis and kinase assay (see Materials and Methods). The reactions contained 40 μg of cytosol proteins and 0.3 μg of phenyl-Superose-purified recombinant $p42^{mapk}$. Autokinase activity without cytosol (–) and cytosol without recombinant substrate (lanes at either end of gel) are also displayed. The data are typical of at least three experiments done with fresh lysates.

blotted either with an antipeptide antibody recognizing $p42^{mapk}$ (Fig. 1, upper panel) or with an antiphosphotyrosine antibody (Fig. 1, lower panel). It can be seen that the wild-type $p42^{mapk}$ displayed phosphorylation on tyrosine, which we have previously shown by phosphopeptide mapping to be on Y-185, the site of regulatory tyrosine phosphorylation (36). This tyrosine phosphorylation occurs by an apparently intramolecular reaction and depends on the intrinsic autokinase activity of the enzyme (51). The kinase-defective mutant K52R was not phosphorylated on tyrosine, reflecting its inability to catalyze the autokinase reaction. Similarly, all the mutants in which Y-185 had been replaced by a nonphosphorylatable amino acid were not tyrosine phosphorylated. These data confirm that phosphorylation depends on the kinase activity of $p42^{mapk}$ and strengthen the conclusion that the tyrosine autophosphorylation is on Y-185.

Mutant kinases in which threonine 183 was replaced by a nonphosphorylatable amino acid still demonstrated autophosphorylation on tyrosine 185. Interestingly, reactivity with antiphosphotyrosine antibodies of the recombinant $p42^{mapk}$ was decreased in the T183A mutant and enhanced in the T183E mutant. This suggests that a negative charge at the position of the regulatory threonine phosphorylation might enhance the autophosphorylation on Y-185. However, it is conceivable that the mutations affect the affinity of the antiphosphotyrosine antibody for the phosphorylated Y-185.

The $p42^{mapk}$ mutants containing glutamate at either T-183 or Y-185 exhibited a decreased mobility on SDS-PAGE. A similar decrease in mobility has been observed upon phosphorylation and activation of $p42^{mapk}$ in response to agonist treatment of quiescent cells (37). However, the mutants with glutamate at these sites of regulatory phosphorylation displayed protein kinase activities (with MBP as a substrate) which were at best only marginally higher than that observed with wild-type recombinant $p42^{mapk}$ and were only about 1% of that found for the phosphorylated and activated $p42^{mapk}$ isolated from stimulated mammalian cells (data not shown). Thus, a mobility shift for this kinase is not necessarily associated with enzymatic activation.

In vitro phosphorylation of recombinant $p42^{mapk}$. To determine whether the intrinsic autokinase activity of $p42^{mapk}$ might play a role in the regulatory phosphorylation of this enzyme in growth factor-stimulated mammalian cells, we compared the ability of cytosols to phosphorylate either wild-type recombinant $p42^{mapk}$ protein or the kinase-defective K52R mutant protein. We reasoned that cellular factors capable of phosphorylating the kinase-defective protein must themselves be kinases, whereas those that require a kinase-competent substrate were candidate enhancers of the intrinsic autokinase activity.

We partially purified wild-type and K52R recombinant $p42^{mapk}$ proteins from bacterial lysates (see Materials and Methods). These proteins were then tested as substrates for in vitro phosphorylation by incubating them in the presence of [γ-^{32}P]ATP with or without unfractionated cytosol prepared from quiescent Swiss 3T3 cells which had been untreated or had been stimulated for 5 min with either PDGF, EGF, or TPA. Total cytosolic extracts were used in these experiments so as to make possible the detection of diverse factors capable of phosphorylating $p42^{mapk}$.

It can be seen (Fig. 2 and 3) that, as expected, the wild-type $p42^{mapk}$ (MK lanes) autophosphorylated even without cytosol. This phosphorylation occurred largely on Y-185 under the conditions used here (51; our unpublished observations). The kinase-defective K52R mutant (KR lanes) did not display detectable autokinase activity.

When incubated for 20 min with cytosol prepared from unstimulated 3T3 cells, the wild-type $p42^{mapk}$ enzyme displayed a modest enhancement of phosphorylation. In contrast, the same amount of kinase-defective K52R mutant was phosphorylated by the cytosolic extracts from unstimulated cells very poorly, if at all. These results could be interpreted as suggesting the presence of an activity in cell cytosols which can cause phosphorylation only of a kinase-active $p42^{mapk}$. This issue will be dealt with in the Discussion.

Cytosolic extracts prepared from cells stimulated with PDGF, EGF, or TPA displayed much greater $p42^{mapk}$ phosphorylating activity than did cytosolic extracts from unstimulated cells. Moreover, in these lysates both the wild-type (MK lanes) and K52R mutant became phosphorylated (Fig. 2 and 3). Thus, agonist stimulation of quiescent cells activates a kinase capable of phosphorylating even the kinase-

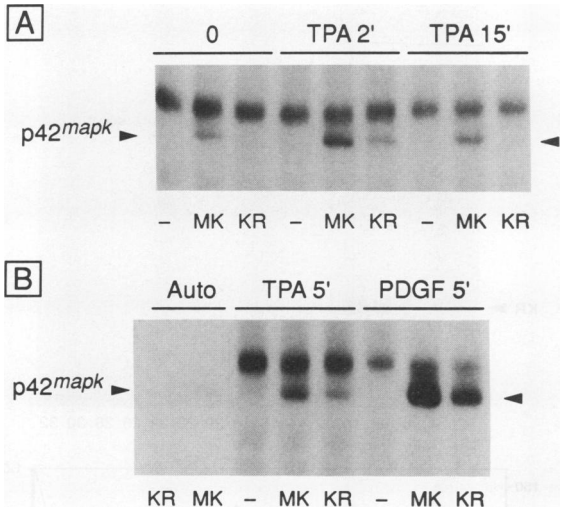

FIG. 3. In vitro phosphorylation of wild-type (MK) and kinase-defective (KR) p42^{mapk} by cytosolic extracts of 3T3 cells activated with PDGF or TPA. After agonist stimulation, cells were lysed in the cell lysis buffer (see Materials and Methods) supplemented with 10 mM NaF and 30 mM β-glycerophosphate and were stored frozen prior to use. Phosphotransferase reactions were performed as described in Materials and Methods with 0.9 μg of phenyl-Sepharose-purified recombinant p42^{mapk} and 12% ethylene glycol. Note that the use of frozen lysates and partially purified p42^{mapk} substrate resulted in less phosphorylation of the p42^{mapk} substrate than in Fig. 2; at the exposure used to reveal p42^{mapk} phosphorylations, the endogenous lysate phosphorylations are more evident. (A) Quiescent cells were stimulated with 125 ng of TPA per ml for 0, 2, or 15 min before lysis. Sixty micrograms of cytosol protein was used. (B) Cells were stimulated for 5 min with 125 ng of TPA per ml or 20 ng of PDGF. Eighty micrograms of cytosol protein was used. The autokinase control (Auto) also pertains to panel A. The relative phosphorylation of MK versus KR is typical of more than four experiments done with lysates which had been frozen.

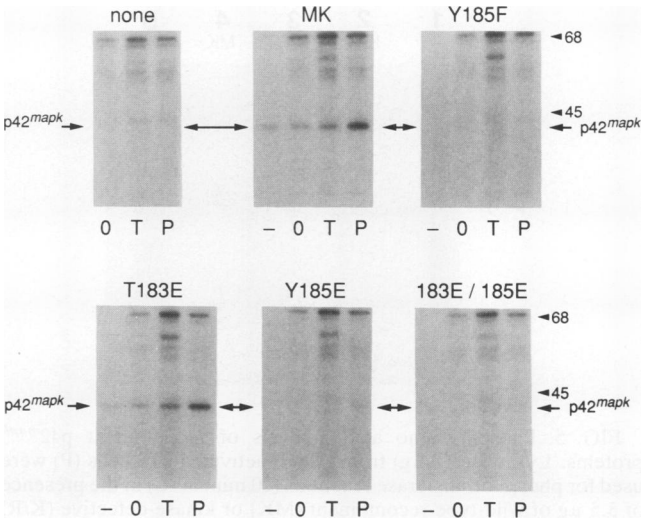

FIG. 4. In vitro phosphorylation of wild-type (MK) and phosphorylation-site mutants of p42^{mapk} by cytosolic extracts of 3T3 cells activated with PDGF or TPA. Cytosolic extracts and kinase assays were as performed as described in the legend to Fig. 3B, except that 25% ethylene glycol and 40 μg of lysates were in the kinase reaction. Assays were done either without added p42^{mapk} (upper left panel), with addition of wild-type p42^{mapk} (MK), or with the addition of the indicated point mutants. The results are representative of three independent experiments. The positions (in kilodaltons) of molecular mass markers are shown to the right of the rightmost panels.

defective p42^{mapk} mutant; that is, a "kinase kinase" is activated by agonist stimulation of these cells. For purposes of clarity, we will refer to this "kinase of p42^{mapk}/MAP kinase" activity as MAPKK, as proposed by Gomez and Cohen (18) without intending to imply whether this represents a single kinase or a mixture of kinases.

MAPKK activity also has been detected in cytosolic extracts from cells in which p42^{mapk} has been activated by treatment with fluoroaluminate, implying that this kinase can be activated via a G-protein-linked pathway (data not shown).

Following stimulation of quiescent cells with TPA, the MAPKK activity appeared rapidly, being detectable at 2 min and declining by 15 min (Fig. 3A). Thus, the MAPKK activity appeared in response to this agonist at least as rapidly as did the activation of p42^{mapk} itself. For this reason, 5 min of stimulation has been used in most of our experiments. Under these conditions, PDGF appears to be the most potent stimulator of MAPKK activity in Swiss 3T3 cells, with TPA and EGF providing less stimulation (Fig. 2 and 3B).

To identify the site(s) of phosphorylation catalyzed by the MAPKK activity, p42^{mapk} proteins carrying mutations in the sites of regulatory phosphorylation were isolated and similarly incubated with cytosols prepared from TPA- or PDGF-treated cells (Fig. 4). It can be seen that changing Y-185 to a nonphosphorylatable amino acid almost completely blocked the susceptibility of p42^{mapk} to phosphorylation in these cytosolic extracts, strongly suggesting that under these conditions the phosphorylation detected was largely on Y-185. Even though phosphorylation of T-183 is also required for p42^{mapk} enzyme activity (7, 35), the data in Fig. 4 imply that T-183 phosphorylation occured only to a small extent in these cytosolic extracts. This conclusion has been confirmed by phosphoamino acid analysis (Fig. 5). The basis for the weak ability of these lysates to stimulate phosphorylation of T-183 is under investigation.

The T183E mutant did not display an enhanced susceptibility to in vitro phosphorylation in cytosol, even though the steady-state tyrosine phosphorylation of this mutant was apparently enhanced when expressed in bacteria (compare Fig. 4 and 1). It is possible that the stoichiometry of basal Y-185 phosphorylation on this mutant protein is sufficiently high so as to reduce its susceptibility to further phosphorylation in vitro.

Phosphoamino acid analysis of in vitro-phosphorylated p42^{mapk}. As mentioned above, the recombinant p42^{mapk} incubated with cytosols from agonist-stimulated cells becomes phosphorylated predominantly on tyrosine, with smaller amounts of phosphothreonine detectable (Fig. 5). This was shown directly by phosphorylating either wild-type or K52R recombinant substrates with cytosolic extracts from PDGF-stimulated cells (P lanes), electrophoresing the reaction mixture, and excising the p42^{mapk} band. In this reaction, a protein of 43 to 44 kDa present in cytosol also became heavily phosphorylated on serine and was excised along with the p42^{mapk} to provide an internal control for recoveries. The phosphoamino acid analysis of this material is shown in lane 1 of Fig. 5 and completely accounts for the phosphoserine obtained in lanes with added p42^{mapk} substrate (lanes 2 to 5). It can be seen that when incubated with

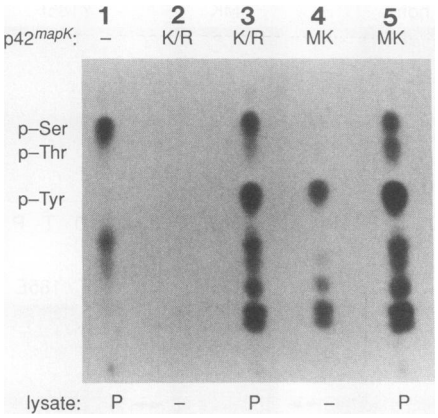

FIG. 5. Phosphoamino acid analysis of recombinant p42^{mapk} proteins. Lysates (150 μg) from PDGF-activated 3T3 cells (P) were used for phosphotransferase reactions (20 min, 30°C) in the presence of 5.5 μg of wild-type recombinant (MK) or kinase-defective (K/R) protein purified with phenyl-Sepharose. Parallel autokinase reactions were also performed. Samples containing the phosphoamino acids were prepared as described in Materials and Methods. Note that a protein of 43 to 44 kDa whose intensity depends on the amount of lysates (compare Fig. 2 and 4 to Fig. 3) becomes phosphorylated specifically on serine in these lysates. This band was excised along with the p42^{mapk} band and used as an internal control.

cytosols from PDGF-treated cells, both the wild-type (MK) and kinase-defective (K/R) proteins became phosphorylated predominantly on tyrosine and to a lesser extent on threonine (lanes 3 and 5). The wild-type protein displayed autokinase activity (under these conditions almost entirely on tyrosine), whereas the kinase-defective protein did not display autokinase activity (lanes 2 and 4).

Enzymatic activation of p42^{mapk}. To determine whether the phosphorylations described above could be accompanied by increased enzymatic activity of p42^{mapk}, we chromatographed cytosolic extracts from EGF-stimulated cells on MonoQ FPLC to resolve the MAPKK activity from enzymatically activated p42^{mapk} present in the agonist-stimulated lysates. The endogenous p42^{mapk} activity is retained on a MonoQ column, eluting around 125 to 150 mM NaCl. However, an activity capable of activating exogenously added recombinant p42^{mapk} was detected in the isocratic wash (Fig. 6B). This ability to enzymatically activate p42^{mapk} comigrated with the bulk of the MAPKK activity which phosphorylated the kinase-defective K52R recombinant p42^{mapk} (Fig. 6A). Lysates from TPA- or PDGF-stimulated cells also showed a MAPKK activity coeluting with the p42^{mapk} activating activity in the isocratic wash from the MonoQ column (data not shown). The activating activity and the kinase kinase activity from the EGF-stimulated cells further copurified on MonoS FPLC (unpublished observations).

MAPKK activity depends on protein phosphorylation. For the first step in understanding the regulation of MAPKK activity, we have examined its sensitivity to inactivation by phosphatase 2A, a serine/threonine phosphatase. Figure 7 shows that the ability of cytosolic extracts from EGF- or PDGF-treated cells to phosphorylate the kinase-defective K52R mutant of p42^{mapk} can be reduced by 5 min of phosphatase treatment and can be completely inactivated by 30 min of treatment. The action of the phosphatase was blocked by the phosphatase inhibitor okadaic acid. Thus, the kinase kinase depends on serine/threonine phosphorylation

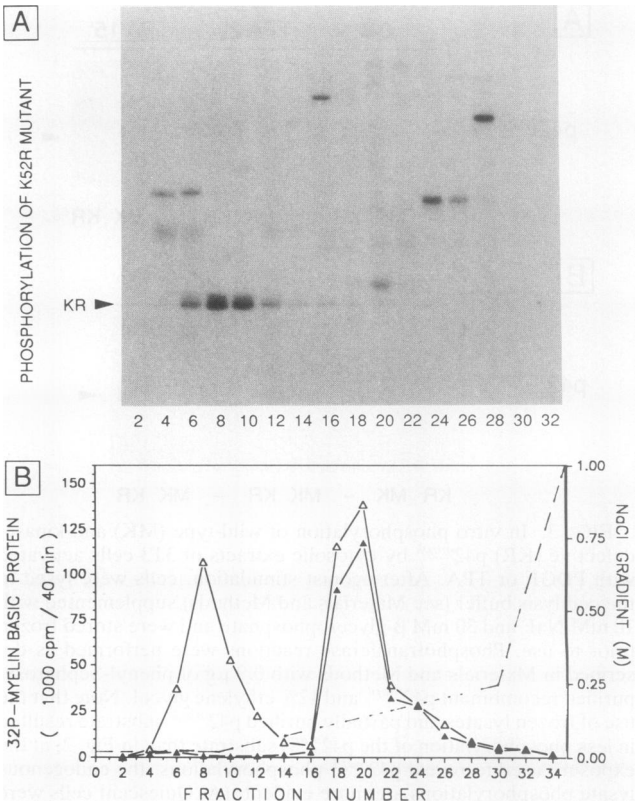

FIG. 6. MonoQ fractionation of cytosol from EGF-activated 3T3 cells. Cytosol from 10 confluent 100-mm-diameter dishes of 3T3 cells (approximately 12 mg of protein) which had been stimulated with 300 ng of EGF per ml for 5 min before lysis was clarified and applied to a MonoQ anion-exchange column as described in Materials and Methods. (A) Phosphorylation of the K52R mutant was performed for 20 min at 30°C as described in Materials and Methods, using 1/50 (20 μl) of the volume fraction and 0.3 μg of phenyl-Superose-purified KR. (B) MBP kinase activity was assessed for 40 min as described in Materials and Methods with 10 μl of each fraction and 0.5 mg of MBP per ml in the presence (△) or absence (▲) of 0.17 μg of MK-B protein. Data are typical of two independent experiments.

and may be regulated in this way in response to agonists. Preliminary data (44a) indicate that the MAPKK activity cannot be inactivated by the tyrosine-specific phosphatase CD45, under conditions which inactivate p42^{mapk}, in agreement with the findings of Gomez and Cohen (18).

DISCUSSION

A kinase which phosphorylates p42^{mapk}. p42^{mapk} is a serine/threonine protein kinase which becomes phosphorylated on tyrosine and threonine and consequently becomes activated in response to diverse agonists. We find that stimulation of cells with various agonists results in the appearance of an activity which can phosphorylate and enzymatically activate p42^{mapk}. Because p42^{mapk} displays a measurable ability to autophosphorylate on the regulatory tyrosine, Y-185, we initially suspected that the activating factor might be a protein which enhanced this intrinsic autokinase activity. However, the predominant activity in cytosols prepared from cells stimulated with various agonists is able to phosphorylate a kinase-defective mutant of p42^{mapk}, indicating

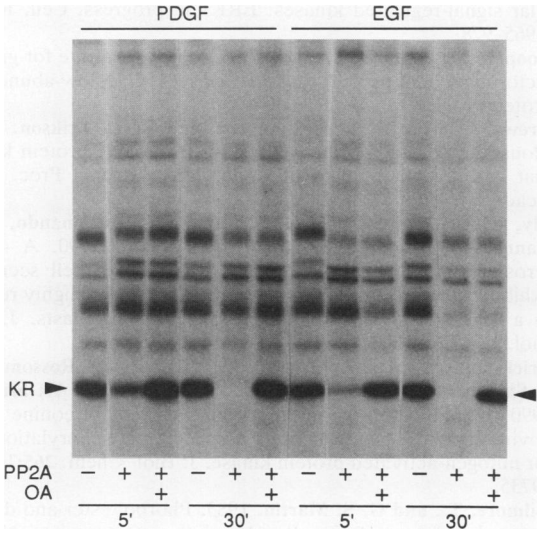

FIG. 7. Inactivation of MAPKK after phosphatase 2A treatment. Ten milligrams of 3T3 cells was activated for 5 min either by 300 ng of EGF per ml or by 10 ng of PDGF per ml, lysed, and fractionated through MonoQ. The eluted fractions were analyzed for their capacity to phosphorylate the K52R mutant (see Materials and Methods). Those containing the activator(s) were pooled and concentrated 40 times from which 2.5 μl (1/40 of recovered volume) was preincubated with 1 μM okadaic acid (OA) (+) or not, and treated (+) or not (−) with 600 U of phosphatase 2A (PP2A) per ml for 5 or 30 min. The reaction was stopped by addition of 1 μM okadaic acid in every sample, and the kinase assay with the phenyl-Superose-purified K52R (KR) was then performed for 20 min as described in Materials and Methods.

that the major activating factor is itself a kinase, in agreement with the suggestion of Gomez and Cohen (18). Posada and Cooper (37) have reached a similar conclusion in their studies with progesterone-stimulated *Xenopus* oocytes. Thus, treatment of quiescent cells with diverse agonists results in the activation of one or more kinase kinases. As proposed by Gomez and Cohen (18), we refer to this activity as MAPKK.

A mutant form of p42^{mapk} in which the regulatory threonine, T-183, had been mutated to glutamate or alanine still exhibited phosphorylation on tyrosine 185, indicating that tyrosine phosphorylation could occur without prior threonine phosphorylation. In addition, the kinase-defective K52R mutant exhibited phosphorylation on both tyrosine and threonine. Thus, both threonine and tyrosine phosphorylations can occur via exogenous kinase activity, and neither is strictly dependent on autophosphorylation.

The MAPKK activity (measured by phosphorylation of a kinase-defective p42^{mapk} mutant substrate) copurified on MonoQ FPLC with activity which caused wild-type recombinant p42^{mapk} to become enzymatically activated, suggesting the identity of these two activities. These activities have continued to copurify over a total of five chromatographic steps (44b). Thus, we propose that the predominant p42^{mapk} activating factor in lysates prepared from cells stimulated with a variety of agonists is a kinase kinase.

The enzymatic activation of p42^{mapk} depends both on threonine and tyrosine phosphorylation. MAPKK is not sufficiently purified to determine whether it consists of a single protein kinase with dual specificity for threonine and tyrosine or whether a mixture of two (or more) kinases is carrying out these regulatory phosphorylations. In addition, it still remains possible that the tyrosine phosphorylation is followed by an intramolecular phosphate transfer, leading to threonine phosphorylation.

An "autokinase-enhancing factor?" In addition to clearly identifying a kinase kinase, we also have obtained evidence suggesting existence of a cellular factor which causes phosphorylation of the wild-type p42^{mapk} but not of the kinase-defective mutant, K52R: we often find that cytosols from both agonist-stimulated and unstimulated cells cause somewhat higher levels of phosphorylation of the wild-type p42^{mapk} than of the kinase-defective K52R mutant. It is possible that this activity reflects the existence of an autokinase-enhancing factor; i.e., a protein which enhances the intrinsic autokinase activity of p42^{mapk} (31).

Although we are attracted to the notion that the intrinsic autokinase activity of p42^{mapk} can be utilized by an autokinase-enhancing factor to achieve physiological regulation of this enzyme, the evidence we have thus far obtained for this factor is not definitive. Even if wild-type p42^{mapk} were more readily phosphorylated by cytosol than the kinase-defective mutant, this result may not be directly related to the kinase activity possessed by the wild-type substrate. For example, it is possible that the K52R mutant protein folds aberrantly in *E. coli* and thus is not as good a substrate for MAPKK as is the wild-type protein. In addition, attempts to identify the enhancing factor activity as a chromatographic peak (distinct from MAPKK) have thus far been unsuccessful. Finally, we have not found conditions of cell growth, stimulation, or extract preparation which would consistently yield cytosols in which the putative autokinase-enhancing factor was the major regulator of p42^{mapk}. For these reasons, we view our data concerning an autokinase-enhancing factor as only suggestive. Additional studies on the cell biology and biochemistry of the factors regulating p42^{mapk} phosphorylation will be necessary to determine whether an autokinase-enhancing factor exists and if so to determine the relative extent to which the different mechanisms of p42^{mapk} activation are utilized in vivo.

p42^{mapk} and the integration of cellular signalling. In our previous publications, we have sometimes referred to p42^{mapk} as a switch kinase, because it functions as a serine/threonine kinase but is regulated by tyrosine phosphorylation (30, 36). We suggested that a number of signalling pathways would converge at p42^{mapk} and that phosphorylation of p42^{mapk} was the point at which these signalling pathways were integrated. In the simplest formulation of this model, p42^{mapk} would be phosphorylated on tyrosine by tyrosine kinase receptors (such as receptors for EGF and PDGF) and would be phosphorylated on threonine by protein kinase C. However, the results presented here demonstrate an additional level of complexity: p42^{mapk} is phosphorylated on tyrosine and threonine by one or more cytosolic kinases whose activity is in turn regulated (directly or indirectly) by the membrane-associated kinases. Thus, MAPKK and other factors which regulate the phosphorylation and activation of p42^{mapk} appear to be the site of signal integration. Moreover, the fact that the MAPKK activity is inactivated by phosphatase 2A but not by CD45 suggests (but does not prove) that this kinase is regulated by serine/threonine phosphorylation and not by tyrosine phosphorylation. Thus, the biochemical pathway leading from the activation of tyrosine kinases such as the EGF and PDGF receptors to the tyrosine phosphorylation of p42^{mapk} may be more indirect than previously supposed. Although substrates for p42^{mapk} are being determined (6, 11, 35, 39–41,

49), the events which lie between membrane function and regulation of this enzyme appear to be quite complex.

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The first two authors contributed equally to this work.

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ADDENDUM IN PROOF

While this manuscript was under review, Ettehadieh et al. (Science **255**:853–855, 1992) reported that a MAP kinase isoform from sea star oocytes could be phosphorylated by the membrane-associated tyrosine kinase p56 ^{lck}. These results contrast with our conclusion that a cytosolic dual specificity MAPKK is the predominant mechanism for regulatory phosphorylation of MAP kinase. However, it is possible that there are alternative pathways for activation of MAP kinases.

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