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Autophosphorylation *in vitro* of recombinant 42-kilodalton mitogen-activated protein kinase on tyrosine

(mitogen-activated protein kinase/cell cycle/peptide mapping/recombinant expression)

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ABSTRACT **Mitogen-activated protein kinase (MAP kinase) is a serine/threonine protein kinase that becomes enzymatically activated and phosphorylated on tyrosine and threonine following treatment of quiescent cells with a variety of stimulatory agonists. Phosphorylation on both tyrosine and threonine is necessary to maintain full activity, and these two regulatory phosphorylations occur close to each other, separated by a single glutamate. To study the mechanisms by which MAP kinase becomes phosphorylated and activated, we have cloned a full-length cDNA encoding MAP kinase and have expressed the enzyme in *Escherichia coli* as a soluble nonfusion protein. We find that the enzyme displays a basal, intramolecular autophosphorylation on tyrosine-185 that is accompanied by activation of the enzyme's kinase activity towards an exogenous substrate. The tyrosine-phosphorylated protein displays a small fraction of the activity seen with the fully activated, doubly phosphorylated enzyme isolated from mammalian cells but is activated 10- to 20-fold relative to the unphosphorylated enzyme. These findings raise the possibility that regulation of MAP kinase activity in response to agonist stimulation could occur in part through the enhancement of autophosphorylation on tyrosine.**

Mitogen-activated protein kinase (MAP kinase) was originally identified as a serine/threonine protein kinase that became phosphorylated on tyrosine and threonine and enzymatically activated following insulin-stimulation of 3T3-L1 cells (1, 2). This enzyme was subsequently found to be identical or closely related to pp42, a protein that becomes tyrosine-phosphorylated in cells treated with various mitogens (3). pp42/MAP kinase recently has been shown to be encoded by a member of a gene family (4–8). Accordingly, we now refer to it as $p42^{mapk}$ to distinguish it from other members of the family, while still retaining recognizable features of the earlier nomenclature. In addition to being phosphorylated and activated during the $G_0 \rightarrow G_1$ transition, $p42^{mapk}$ and/or members of this family become phosphorylated and activated during M phase in *Xenopus* oocytes and in various differentiated, nonmitogenic cells following treatment with stimulatory agonists (9–11). Therefore, we suspect that this enzyme plays a fundamental role in some process common to a number of regulatory events (for review, see ref. 11).

Phosphorylation of $p42^{mapk}$ on both tyrosine and threonine is required for it to display full enzymatic activity (12). The sites of regulatory phosphorylation were identified by mass spectrometry and found to be on a single peptide separated by only one amino acid, glutamic acid, in a sequence (Thr-Glu-Tyr-Val-Ala-Thr-Arg) that is absolutely conserved in the related protein kinases ERK1 (extracellular signal-related protein kinase 1), KSS1, and FUS3 (13). These phosphorylation sites are located just upstream of the Ala-Pro-Glu motif in a region where activating autophosphorylations occur in many other kinases (5, 6, 14).

To investigate the mechanism(s) for $p42^{mapk}$ activation, we have cloned a full-length cDNA encoding $p42^{mapk}$ (6) and expressed the enzyme in *Escherichia coli* in soluble form. We now report that recombinant $p42^{mapk}$ is basally active and autophosphorylates on tyrosine. This autophosphorylation can occur on the site of regulatory phosphorylation and is accompanied by a partial enzymatic activation of $p42^{mapk}$. Thus, upstream components regulating MAP kinase activity may conceivably do so by enhancing the rate of autophosphorylation on tyrosine.

MATERIALS AND METHODS

Materials. The bacterial expression vector pET11a and the *E. coli* strains HB101 and BL21(DE3) (15) were purchased from Novagen (Madison, WI). Isopropyl β-D-thiogalactopyranoside (IPTG) and enzymes used in DNA manipulations were obtained from GIBCO/BRL. The sources for chromatography media and reagents for culturing *E. coli* were Pharmacia-LKB and Difco, respectively. Oligonucleotides were purchased from Operon Technologies (Alameda, CA), and used without further purification. A synthetic peptide (Val-Ala-Asp-Pro-Asp-His-Asp-His-Thr-Gly-Phe-Leu-Thr-Glu-Tyr-Val-Ala-Thr-Arg) corresponding to the tryptic peptide containing the regulatory phosphorylation sites of $p42^{mapk}$ was purchased from Synthecell (Rockville, MD). Antibody 1798, which recognizes $p42^{mapk}$, was raised against the carboxyl-terminal 10 amino acids of the predicted ERK1 sequence (5) by the MAPS procedure (16).

Construction of Plasmids for Expression of Unfused $p42^{mapk}$ in *E. coli*. Plasmid pET11a was digested with *Nde* I, and the recessive 3′ end was filled in with Klenow fragment. The DNA was then cut with *Bam*HI, generating a 5639-base-pair (bp) fragment, which was isolated by gel electrophoresis. The blunt end of the purified fragment starts at the first nucleotide of the ATG start codon of the phage T7 φ10 gene. The $p42^{mapk}$ cDNA was excised from plasmid pA4 (6) by sequential treatment with *Bgl* I, mung bean nuclease, and *Bam*HI. The ≈1.25-kilobase (kb) $p42^{mapk}$ cDNA fragment was ligated to the above 5639-bp fragment of pET11a and transformed into *E. coli* HB101. Plasmids in which the second nucleotide of the $p42^{mapk}$ cDNA were ligated directly to the first nucleotide of the phage T7 φ10 gene on pET11a reconstitute a translational start codon ATG and thus an unfused $p42^{mapk}$ coding se-

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Abbreviations: MAP kinase, mitogen-activated protein kinase; $p42^{mapk}$, 42-kDa MAP kinase; IPTG, isopropyl β-D-thiogalactopyranoside; MBP, myelin basic protein; ERK1, extracellular signal-related protein kinase 1.

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quence under the control of T7 transcriptional and translational control signals. One such plasmid, pET-MK, was isolated and transformed into expression host *E. coli* BL21(DE3). The kinase-defective Lys-52 → Arg mutant was generated using the Promega "Altered Sites" *in vitro* mutagenesis kit.

Expression of p42^{mapk} in *E. coli*. A 10-ml overnight culture of *E. coli* BL21(DE3)[pET-MK] was inoculated into 1 liter of Luria–Bertoni medium (10% tryptone/5% yeast extract/10% NaCl) containing 0.1 mg of ampicillin per ml. The bacteria were grown at 37°C with vigorous shaking to late logarithmic phase (OD_{600} = 0.8). IPTG was then added to a final concentration of 0.4 mM, and the cells were induced for 4–5 hr before harvesting by centrifugation. For labeling of the phosphorylated p42^{mapk} synthesized in *E. coli*, 0.2 mCi (1 Ci = 37 GBq) of $^{32}P_i$ per ml was added to the bacteria prior to the IPTG induction. The bacterial pellets were frozen at −70°C.

Purification of p42^{mapk} Produced in *E. coli*. Bacterial pellets were thawed, resuspended in 25 mM Tris·HCl, pH 7.5 (4°C)/40 mM *p*-nitrophenyl phosphate/2 mM EGTA/2 mM EDTA/1 mM dithiothreitol/1 mM phenylmethylsulfonyl fluoride and lysed by three passages through a chilled French pressure cell. The cell extracts were centrifuged at 12,000 × *g* for 20 min at 4°C. p42^{mapk} in the supernatant was then purified sequentially by batch absorption to phenyl-Sepharose/Mono Q chromatography and phenyl-Superose chromatography as described (13).

Protein Kinase Assay. Autophosphorylation of recombinant p42^{mapk} was performed by incubation in "autokinase buffer" [25 mM Hepes/10 mM $MgCl_2$/2 mM $MnCl_2$/1 mM dithiothreitol, pH 7.5/50 μM [γ-^{32}P]ATP (5000 cpm/pmol)] at 30°C. The amounts of protein and the time of autophosphorylation are specified in the figure legends. The reaction was stopped by adding SDS electrophoresis sample buffer containing 100 mM EDTA and heating at 95°C for 3 min. Phosphorylated proteins were resolved by electrophoresis in SDS/10% polyacrylamide gels. After autoradiography, phosphorylated p42^{mapk} bands were excised for further analysis.

The synthetic peptide containing the regulatory phosphorylation sequence was phosphorylated with purified pp60^{src} (provided by Nick Lydon, CIBA–Geigy) and purified by reverse-phase HPLC. Incorporation was exclusively on tyrosine, as determined by phosphoamino acid analysis.

Other Methods. Immunoblotting, phosphoamino acid analysis, and phosphopeptide mapping were performed as described (13, 17).

RESULTS

Tyrosine Phosphorylation of Recombinant p42^{mapk} in *E. coli*. We constructed a plasmid (pET-MK) in which p42^{mapk} could be expressed in *E. coli* as a complete, nonfusion protein, inducible by IPTG as described. IPTG induction of *E. coli* carrying pET-MK resulted in the appearance of a 42-kDa protein in the bacterial extract, which was not seen in extracts of bacteria carrying the pET plasmid without the p42^{mapk} insert (data not shown). Immunoblotting of the bacterial lysates with anti-peptide antibody 1798, which recognizes p42^{mapk}, confirmed the inducible expression of this protein in cells carrying the pET-MK plasmid but not in cells with the empty plasmid (Fig. 1 *Upper*). Surprisingly, immunoblotting these lysates with anti-phosphotyrosine antibody revealed that a portion of the p42^{mapk} produced in *E. coli* was phosphorylated on tyrosine (Fig. 1 *Lower*).

Since tyrosine kinases have not been identified in *E. coli*, and the regulatory phosphorylations detected in p42^{mapk} occur in a region (13) where autophosphorylation is known to occur in other kinases (14), we suspected that tyrosine phosphorylation of p42^{mapk} in *E. coli* could be occurring via

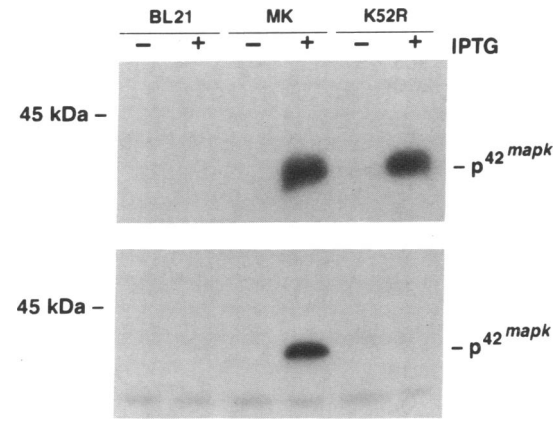

FIG. 1. Expression and tyrosine phosphorylation of p42^{mapk} in bacteria. Wild-type p42^{mapk} (lanes MK) and the kinase-defective Lys-52 → Arg mutant (lanes K52R) were expressed in *E. coli* by induction with IPTG for 4 hr. The bacterial lysates were separated by SDS/PAGE, transferred to nitrocellulose, and blotted with either anti-p42^{mapk} antibody 1798 (*Upper*) or anti-phosphotyrosine antibody (*Lower*).

an autophosphorylation reaction. To test this hypothesis, we generated and expressed a kinase-defective mutant of p42^{mapk}, with a substitution of arginine for lysine at position 52 (in the ATP binding site) (6). Although IPTG also induced the appearance of this mutant protein (Fig. 1 *Upper*), the protein did not become detectably phosphorylated on tyrosine (Fig. 1 *Lower*). Thus, the appearance of phosphotyrosine on bacterially expressed p42^{mapk} requires a kinase-active enzyme, indicating that this tyrosine phosphorylation is a consequence of autophosphorylation.

Purification of Two Forms of p42^{mapk}. Most if not all of the p42^{mapk} produced in *E. coli* was found in the soluble fraction of the cell extract (data not shown). Chromatography on phenyl-Superose reproducibly resolved the recombinant protein into two highly purified forms (estimated purity, >98%): one form was retained on the column and was eluted as a broad peak in the wash (form A) and the other form was eluted during the ethylene glycol gradient (form B). A small portion of the p42^{mapk} appeared in the column flow-through with bacterial proteins and was not further characterized. Silver-stained SDS gels of the peak fractions of forms A and B are shown in Fig. 2 *A* and *B*, respectively. That both forms are in fact p42^{mapk} is demonstrated by immunoblotting of the preparations with anti-p42^{mapk} antibody 1798 (Fig. 2*C*). Form A represents 25–45% of the purified protein (data not shown).

Immunoblotting with anti-phosphotyrosine antibody revealed that the p42^{mapk} form A contained phosphotyrosine but that form B did not (Fig. 2*D*). To determine whether these p42^{mapk} forms might contain other phosphoamino acids, we labeled the recombinant enzyme with ^{32}P by growing the bacteria in the presence of $^{32}P_i$ and then took the radiolabeled extract through the purification procedure. No detectable radioactivity was found in the isolated form B, and only phosphotyrosine was found in form A (data not shown).

***In Vitro* Phosphorylation of p42^{mapk}.** Incubation with [γ-^{32}P]ATP of purified p42^{mapk} revealed *in vitro* incorporation of ^{32}P into the wild-type form of the protein [as described previously by Boulton *et al.* (4)] but not into the kinase-defective mutant form of the protein (data not shown). This demonstrates that the *in vitro* autokinase reaction also depends on the p42^{mapk} kinase activity.

In vitro autokinase activity was detected in purified form A and form B p42^{mapk}. Phosphoamino acid analysis of *in vitro* phosphorylated p42^{mapk} showed that the enzyme could incorporate phosphate onto both tyrosine and threonine (Fig. 3). With the form B enzyme, phosphorylation on tyrosine

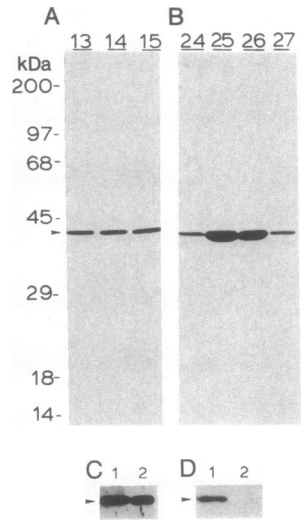

FIG. 2. Purification and immunoblotting of two conformers of p42^{mapk} produced in *E. coli*. (*A* and *B*) Supernatant from IPTG-induced *E. coli* containing the pET-MK plasmid was subjected to chromatography, and fractions from phenyl-Superose were separated by SDS/PAGE. p42^{mapk} peak fractions eluted from phenyl-Superose in the wash (*A*) and at 37% ethylene glycol (*B*) are shown. (*C*) Immunoblot with anti-p42^{mapk} antibody 1798 of pooled wash fractions (form A) or gradient fractions (form B) are shown in lanes 1 and 2, respectively. (*D*) Immunoblot with an anti-phosphotyrosine antibody of the same fractions shown in *C*.

occurred most rapidly and phosphorylation on threonine occurred after a lag; however, little or no lag was observed in the phosphorylation of threonine by form A (which already contains phosphotyrosine). These findings suggest that the autophosphorylation on tyrosine can facilitate the ability of p42^{mapk} to phosphorylate on threonine.

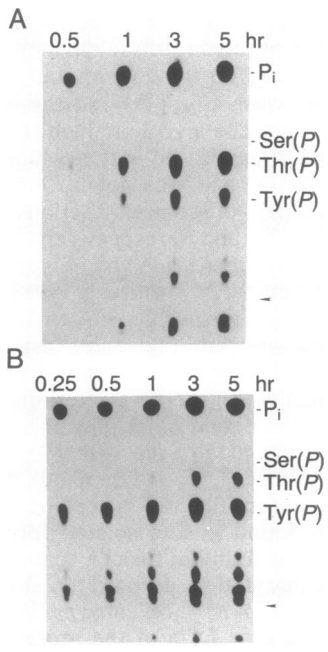

FIG. 3. Phosphoamino acid analysis of *in vitro* phosphorylated p42^{mapk}. Purified form A of p42^{mapk} (*A*) and form B of p42^{mapk} (*B*) were autophosphorylated as described. At indicated time points, aliquots of the reaction mixture containing 0.84 μg of p42^{mapk} were removed, mixed with SDS sample buffer containing EDTA, and boiled. Following electrophoresis and autoradiography, p42^{mapk} bands were excised. The phosphoamino acid content of each excised p42^{mapk} band was then analyzed as described. Ser(*P*), phosphoserine; Thr(*P*), phosphothreonine; Tyr(*P*), phosphotyrosine.

Concentration Dependence of p42^{mapk} Autophosphorylation on Tyrosine. To determine whether the *in vitro* autophosphorylation on tyrosine occurred by an intramolecular vs. intermolecular mechanism, the concentration dependence of the *in vitro* autokinase reaction was measured. For these experiments, we utilized form B of the purified p42^{mapk} and incubated for only 8 min, so that phosphorylation occurred solely on tyrosine, as shown in Fig. 3. The rate of autophosphorylation of p42^{mapk} was found to be independent of concentration (Fig. 4). Thus, the observed endogenous tyrosine phosphorylation of the purified p42^{mapk} appears to be an intramolecular reaction (18). However, it is possible that the threonine phosphorylation occurs by an intermolecular reaction.

Peptide Mapping of *in Vitro* Autophosphorylation Sites. After autophosphorylation *in vitro*, form A contained at least four radioactive tryptic phosphopeptides (Fig. 5*A*), whereas form B contained only a single phosphopeptide (Fig. 5*B*, arrow) that comigrated with a phosphopeptide identified in form A (Fig. 5*C*, arrow). The form B phosphopeptide also comigrated with a synthetic peptide corresponding to the regulatory peptide labeled at Tyr-185 by phosphorylation with pp60^{src} (data not shown). Thus, an exchange reaction is one explanation for the observed tyrosine phosphorylation detected *in vitro* at later times (Fig. 3*A*). Importantly, no phosphopeptide comigrating with the regulatory peptide phosphorylated on both Thr-183 and Tyr-185 was detected in digests of either form phosphorylated *in vitro* (unpublished data). Thus, recombinant p42^{mapk} apparently undergoes intramolecular autophosphorylation at only one (Tyr-185) of the two regulatory sites we previously identified (9).

Myelin Basic Protein (MBP) Kinase Activity of Recombinant p42^{mapk}. Purified form A of p42^{mapk}, which is phosphorylated on tyrosine, was 10–20 times more active than the unphosphorylated form B (≈830 pmol/min per mg of p42^{mapk} vs. 60 pmol/min per mg, respectively) with MBP as an exogenous substrate. To determine whether *in vitro* autophosphorylation on tyrosine could enhance the MBP kinase activity of p42^{mapk}, purified form B was incubated with ATP under autokinase conditions for various times, and the MBP kinase activity was then determined (Fig. 6). Autophosphorylation on tyrosine was accompanied by an increase in the MBP kinase activity of the form B p42^{mapk} (Fig. 6*B*). By contrast, activity of the form A enzyme, which already contains phosphotyrosine, did not increase during this preincubation (Fig. 6*A*) despite incorporation of phosphate into one or more unidentified threonine residues (Fig. 3*A*). This finding is consistent with the results of phosphopeptide mapping which failed to detect the doubly

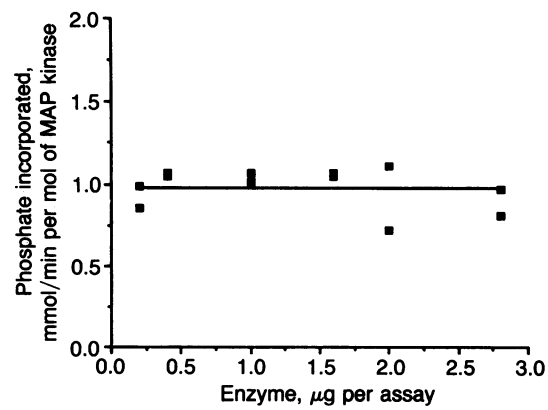

FIG. 4. Effect of enzyme concentration on the initial rate of p42^{mapk} tyrosine autophosphorylation. Duplicate 0.2- to 2.8-μg samples of purified recombinant p42^{mapk} (form B) were autophosphorylated at 30°C for 8 min. The rate of autophosphorylation was determined by liquid scintillation counting of individual p42^{mapk} bands excised from a SDS/10% polyacrylamide gel.

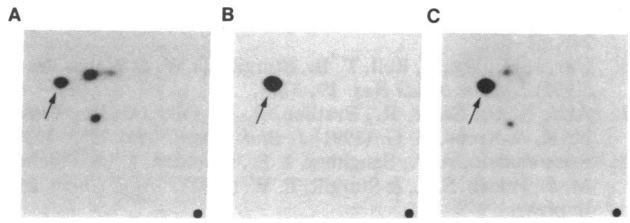

FIG. 5. Peptide mapping of p42^{mapk} autophosphorylation sites. Approximately 0.5 μg of purified form A (*A*) or form B (*B*) of p42^{mapk} were autophosphorylated *in vitro* for 2.5 hr with [γ-^{32}P]ATP followed by proteolysis with trypsin. (*C*) Mixture of the form A and form B phosphopeptides. The origins are indicated by a "dot" in the lower right corner of each panel. Electrophoresis was at pH 1.9 in the horizontal dimension, with the anode at the right.

phosphorylated regulatory peptide in either form of recombinant p42^{mapk} phosphorylated *in vitro*.

DISCUSSION

Although p42^{mapk} was first described as a serine/threonine-specific protein kinase (1), our results demonstrate that this enzyme is capable of autophosphorylation on tyrosine. A kinase-defective mutant of p42^{mapk} did not display autokinase activity, and the autophosphorylation was seen in highly purified preparations of the bacterially expressed enzyme, thus showing that bacterial kinases are unlikely to be responsible for these results. The autokinase activity was linear with enzyme dilution, which is consistent with the finding that this reaction was an autophosphorylation and in addition shows that the tyrosine phosphorylation was intramolecular. Recombinant p42^{mapk} also displayed endogenous phosphorylation on threonine, but it is not known whether this was due to intramolecular or intermolecular reactions, or to both. The tyrosine phosphorylation occurs on the previously identified site of regulatory phosphorylation (13), but the one or more sites of threonine phosphorylation are as yet uncharacterized.

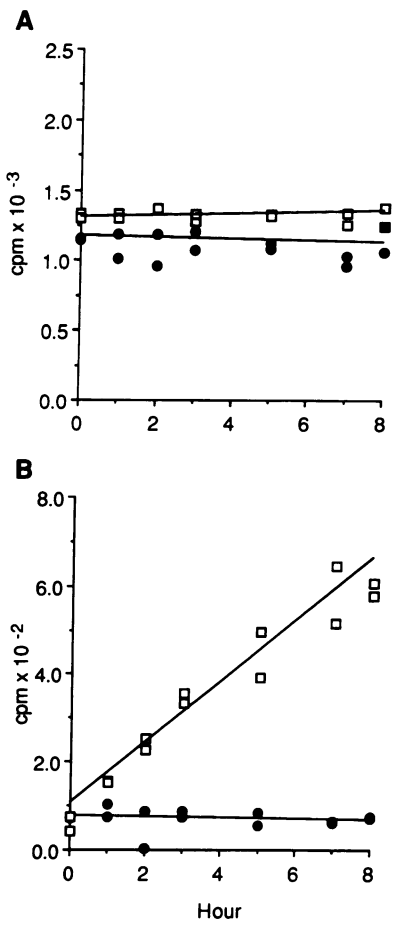

FIG. 6. Partial activation of p42^{mapk} by autophosphorylation. Purified p42^{mapk} (0.4 μg) was incubated in 80 μl of autokinase buffer containing 0.5 mg of bovine serum albumin per ml with (□) or without (●) 50 μM ATP at 30°C for 0–8 hr. At the indicated times, duplicate aliquots (containing 25 ng of p42^{mapk}) were removed and added to MBP kinase assay mixture {25 mM Hepes, pH 7.5/8 μg of MBP/10 mM $MgCl_2$/50 μM (3000 cpm/pmol) [γ-^{32}P]ATP (final concentrations) in a total volume of 40 μl}. The MBP kinase assay was for 20 min at 30°C. Incorporation of radioactivity into MBP was measured by scintillation spectrometry of MBP bands excised from SDS/polyacrylamide gels. (*A*) Form A of p42^{mapk}. (*B*) Form B of p42^{mapk}.

The ability of p42^{mapk} to undergo endogenous phosphorylation on tyrosine and threonine suggests a relationship between this enzyme and a class of recently discovered kinases capable of protein phosphorylation on tyrosine and on threonine/serine (19–22). Indeed, inspection of amino acid sequences (Table 1) in a region of the catalytic domain called subdomain VIII, which is indicative of protein kinase amino acid specificity (14), reveals that p42^{mapk} and the related KSS1 (23), FUS3 (24), and ERK1 (5) kinases share sequence similarities with some of the dual-specificity kinases: just amino-terminal to the conserved Ala-Pro-Glu sequence is an Arg-Zaa-Tyr-Arg sequence, where Zaa is an amino acid with a hydrophobic, planar side group. It will be important to determine whether these related kinases also are capable of autophosphorylation on tyrosine. Site-directed mutagenesis could help determine whether these residues play a role in the specificity of these kinases.

It is important to note that although p42^{mapk} is capable of autophosphorylating on tyrosine, we have no evidence suggesting tyrosine phosphorylation of an exogenous substrate by this enzyme.

In previous studies with p42^{mapk} purified from cultured cells, we failed to detect autophosphorylation. We now suspect that this was a consequence of the very small amounts of enzyme available from the cultured cells and the very slow rate at which the autophosphorylation occurs. In reexamining this issue, we find that small amounts of $^{32}P_i$ can be incorporated into tyrosine in p42^{mapk} isolated from mammalian cells when the enzyme is treated with the tyrosine phosphatase CD45 (25) and subsequently incubated with vanadate and [γ-^{32}P]ATP/Mn (N. G. Anderson and T.W.S., unpublished data).

The bacterially expressed p42^{mapk} could be fractionated by phenyl-Superose chromatography into two purified forms. Form A contained phosphotyrosine while form B was un-

Table 1. Amino acid sequences for subdomain VIII of the catalytic domain of serine/threonine and dual-specificity protein kinases

Protein kinase	Amino acid sequence
MAPK (6)	A T R W Y R A P $^{195}_{202}$E
ERK1 (5)	A T R W Y R A P $^{E}_{195}$
KSS1 (23)	A T R W Y R A P $^{E}_{192}$
FUS3 (24)	A T R W Y R A P $^{E}_{348}$
Clk/STY (19, 21)	S T R H Y R A P $^{E}_{209}$
MCK (20)	C S R F Y R A P E
Consensus	X $^{T}_{S}$ R Z Y R A P E
Ser/Thr consensus (14)	G $^{T}_{S}$ X X $^{Y}_{F}$ X A P E
cAMP-dependent kinase (14)	G T P E Y L A P E

MAPK, MAP kinase.

phosphorylated. Form A displayed an MBP kinase activity 10–20 times higher than that observed with the unphosphorylated form B (although still less than 1% of the specific enzyme activity observed with the fully activated and dually phosphorylated enzyme isolated from agonist-treated cultured cells). Incubation of form B with ATP resulted in autophosphorylation on tyrosine on the site of regulatory phosphorylation seen *in vivo* (13) and in partial activation of MBP kinase activity. Thus, autophosphorylation of $p42^{mapk}$ on tyrosine is associated with enhanced enzymatic activity.

Because the autophosphorylation of $p42^{mapk}$ on tyrosine is accompanied by enhanced enzymatic activity and occurs on a site of regulatory phosphorylation, autophosphorylation is a plausible mechanism by which the activating tyrosine phosphorylation of $p42^{mapk}$ could occur *in vivo* in response to agonists. Although the basal rate of autophosphorylation is too slow to account for the rapid activation that occurs in mammalian cells treated with mitogens, a function of upstream factors regulating $p42^{mapk}$ (7) may be to accelerate the rate of autophosphorylation on Tyr-185, thus contributing to enzymatic activation. Whether phosphorylation of Thr-183 also can occur by an autokinase mechanism or whether this phosphorylation requires another kinase is currently unknown.

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Note Added in Proof. After submission of this paper for review, Seger *et al.* (26) also reported endogenous phosphorylation of $p42^{mapk}$ on tyrosine and threonine, consistent with the findings herein.

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