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Identification of Tyr-185 as the site of tyrosine autophosphorylation of recombinant mitogen-activated protein kinase p42^{mapk}

(mass spectrometry/peptide mapping)

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ABSTRACT **Tyrosine phosphorylation of 42-kDa mitogen-activated protein kinase (p42^{mapk}) occurs during expression of the recombinant protein in *Escherichia coli*, as well as during *in vitro* phosphorylation of the protein purified from this source. Structural analyses were performed to identify the site(s) of tyrosine phosphorylation of recombinant p42^{mapk}, both during expression of the protein in *E. coli* and during *in vitro* incubations with ATP/Mg^{2+}/Mn^{2+}. Mass spectrometry and phosphopeptide mapping showed that tyrosine phosphorylation of recombinant p42^{mapk} occurs on Tyr-185, the site of regulatory tyrosine phosphorylation that occurs in mitogen-stimulated mammalian cells.**

p42^{mapk}, a 42-kDa mitogen-activated protein (MAP) kinase, is rapidly activated in quiescent cells in response to diverse peptide and nonpeptide mitogens (reviewed in ref. 1). Activation of p42^{mapk} occurs by dual phosphorylations of the enzyme at Thr-183 and Tyr-185 (2–4). The activated enzyme appears to function in one or more protein kinase cascades important for transducing mitogenic signals by phosphorylation of downstream substrates. The epidermal growth factor receptor (5), the serine/threonine kinase c-Raf (6), the transcription factor c-Jun (7), and members of the 90-kDa family of S6 kinases (1) have been implicated as probable substrates for p42^{mapk}.

p42^{mapk} is now appreciated as a member of a family of protein kinases that are regulated by tyrosine and serine/threonine phosphorylation, with functions in signal transduction and cell cycle control. Biochemical characterizations have revealed that mitogens stimulate two related MAP-2/MBP kinases (so called because of their ability to phosphorylate microtubule-associated protein 2 and myelin basic protein *in vitro*) in quiescent cells, MBP kinase I (8)/p42^{mapk} (9) and MBP kinase II (8)/p44^{mapk} (9). cDNAs for family members have been isolated from several species (4, 10–12). The first cDNA (erk1) encoding a MAP kinase family member was isolated from a rat library by Boulton *et al.* (10), and encodes a polypeptide of 44–45 kDa by SDS gel mobility, probably corresponding to p44^{mapk}. Recently we isolated a cDNA for p42^{mapk} from a Swiss mouse 3T3 library (4) and found it to be identical, at the amino acid level, to another rat cDNA (erk2) isolated by Boulton *et al.* (11). p42^{mapk}/ERK2 and p44^{mapk}/ERK1 are closely related by sequence to each other (10–12), and both are activated by tyrosine and threonine phosphorylations (2, 9, 11). Together they account for two 41- to 45-kDa proteins previously identified as the principal tyrosine-phosphorylated proteins rapidly appearing in the cytosol of mitogen-treated fibroblasts (e.g., ref. 13).

Although p42^{mapk} phosphorylates the identified exogenous substrates [microtubule-associated protein 2 (14), MBP (15), c-Jun (7), S6 kinase II (16)] on serine/threonine residues, purified recombinant MAP kinase undergoes endogenous phosphorylation on tyrosine and threonine residues (17–19). We now report structural studies that definitively establish the site of tyrosine autophosphorylation in recombinant MAP kinase as Tyr-185 by use of complementary peptide mapping and mass spectrometry techniques. By consideration of the crystal structure deduced for the catalytic subunit of cAMP-dependent protein kinase (20), Tyr-185, as well as Thr-183, is located near the catalytic fold. This comparison suggests, in broad terms, a molecular mechanism for activation of p42^{mapk}.

MATERIALS AND METHODS

Materials. A synthetic peptide (VADPDHDHTGFLTEY-VATR) corresponding to the tryptic peptide containing the regulatory phosphorylation sites of p42^{mapk} (3) was purchased from Synthecell (Rockville, MD). Residues 13 and 15 (in TEY) of this peptide correspond to Thr-183 and Tyr-185 of p42^{mapk} (4); the latter residue names will be used in reference to the peptide as well as the parent protein. p42^{mapk} was expressed as a soluble nonfusion protein in *Escherichia coli* and purified by published procedures (19).

Tryptic Digestion for Mass Spectrometry. One hundred to 150 pmol of recombinant p42^{mapk} in 40 μl of 25 mM Tris, pH 7.5/2 mM EGTA/40 mM *p*-nitrophenyl phosphate/1 mM dithiothreitol/25 mM NaCl/100 mM NH_4HCO_3/1 mM $CaCl_2$ was digested with sequencing-grade trypsin (Boehringer Mannheim). Trypsin (0.3 μg) was added for 5 hr at 37°C. After addition of another 0.2 μg of trypsin, the digestion was completed at room temperature for 12 hr. The reaction was stopped by addition of acetic acid [final concentration, 5% (vol/vol)].

Mass Spectrometry. Mass spectra were recorded on a TSQ-70 triple-quadrupole instrument (Finnigan–MAT, San Jose, CA) equipped for electrospray ionization. Aliquots from the total digest, dissolved in 5% acetic acid, were injected into the electrospray ionization source from a fused-silica, microcapillary HPLC column [100 μm (i.d.) × 70 cm] containing C_{18} packing material in the terminal 15 cm (21). Peptides were eluted with a 10-min 0–80% gradient of aqueous 0.5% acetic acid in acetonitrile, at 1 μl/min.

Phosphopeptide Mapping. Phosphorylation of recombinant p42^{mapk} with [γ-^{32}P]ATP/Mg^{2+}/Mn^{2+} *in vitro* was performed (19) for 2.5 hr. Ten to 15 pmol of recombinant ^{32}P-labeled p42^{mapk} was electrophoresed in an SDS/10% polyacrylamide gel. p42^{mapk} was identified by autoradiography and eluted from the gel into 25 mM *N*-ethylmorpholine (pH 7.7). Tryptic

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Abbreviations: CAD, collision-activated dissociation; MAP kinase, mitogen-activated protein kinase; MBP, myelin basic protein.

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digestion of the eluted protein, phosphopeptide mapping, and phospho amino acid analyses were performed as described by Erickson *et al.* (15).

Other Methods. The synthetic peptide was specifically phosphorylated on the single tyrosine residue by purified $pp60^{src}$ (kindly provided by Nick Lydon, Ciba–Geigy) and [γ-^{32}P]ATP/Mg^{2+} and was purified by reverse-phase chromatography. ^{32}P-labeled regulatory peptide containing phosphotyrosine and phosphothreonine was prepared from ^{32}P-labeled $p42^{mapk}$ purified from phorbol ester-stimulated EL4 cells (3).

RESULTS

A significant fraction (20–30%) of wild-type $p42^{mapk}$ becomes phosphorylated on tyrosine, as assessed by immunoblotting, when expressed in *E. coli*, whereas a mutant (K52R) bearing a Lys → Arg mutation of the active-site residue Lys-52 does not (19). When the recombinant $p42^{mapk}$ was purified, a peak of tyrosine-phosphorylated $p42^{mapk}$ was resolved from a peak lacking phosphotyrosine immunoreactivity (19). $p42^{mapk}$ containing phosphotyrosine (defined as form A) was eluted in the isocratic wash, whereas $p42^{mapk}$ lacking phosphotyrosine (defined as form B) was eluted during the gradient. The specific MBP kinase activity of form A was 10- to 20-fold greater than that of form B; consequently, structural studies were performed to determine the site(s) of tyrosine phosphorylation.

Mass Spectrometry of Recombinant $p42^{mapk}$ from *E. coli*. Purified forms A and B of $p42^{mapk}$ were each treated with trypsin and analyzed by mass spectrometry. Aliquots of the digests were separated on a C_{18} reverse-phase microcapillary HPLC column, and the eluted peptides were directly injected into the electrospray ionization source for mass spectral analysis (see *Materials and Methods*). Phosphopeptides are readily identified by mass spectrometry because each phosphoester formed by transfer of phosphate from ATP to Ser, Thr, or Tyr acceptors increases the mass of the corresponding peptide by 80 Da. The predicted m/z value for the $(M + H)^+$ ion corresponding to the tryptic peptide VADPDHDHTGFLTEYVATR containing the regulatory phosphorylation sites is 2145 (average mass). The electrospray ionization spectrum of form B shows signals at m/z 1073, 716, and 537 (data not shown). These latter ions correspond to $(M + 2H)^{2+}$, $(M + 3H)^{3+}$, and $(M + 4H)^{4+}$, respectively. The electrospray ionization spectrum recorded on form A of the same peptide shows ions corresponding to $(M + 2H)^{2+}$, $(M + 3H)^{3+}$, and $(M + 4H)^{4+}$, at m/z 1113, 742, and 557, respectively (data not shown). The mass of the $(M + H)^+$ ion for form A calculated from these values is 2225 (average mass). The mass difference between the two peptides in form A versus form B is 80 Da. We conclude that one residue in this peptide is phosphorylated in form A. A small amount (<10% of the phosphorylated form) was detected in form B of the protein, as a result of "tailing" of the A form during phenyl-Superose chromatography. No other phosphorylated peptide has been found as yet by mass spectral analysis, in agreement with other methods used to assess the presence of phosphorylated residues (see below).

To determine the site of phosphorylation, a collision-activated dissociation (CAD) mass spectrum was recorded on $(M + 3H)^{3+}$ ions (m/z 742) generated in the electrospray ionization spectrum of the phosphorylated tryptic peptide from form A of $p42^{mapk}$. An aliquot (5 pmol) of the total tryptic digest was applied to a C_{18} microcapillary HPLC column and peptides in the sample were eluted directly into the electrospray ionization source. At the elution time of the phosphorylated tryptic peptide, $(M + 3H)^{3+}$ ions (m/z 742) were selected in the first quadrupole and subjected to CAD. The resulting CAD spectrum and the sequence of amino acids deduced from it are shown in Fig. 1. Predicted monoisotopic masses for fragment ions of type *b* and type *y* (22) are shown above and below the structure in Fig. 1. Those observed in the spectrum are underlined. As detailed below, the data demonstrate that phosphorylation is on Tyr-185.

Fragments of type *y*, both singly and doubly charged, dominated the CAD spectrum (Fig. 1). These ion types all contain the carboxyl-terminal residue plus 1, 2, 3, etc. additional residues (22). Subtraction of m/z values for any two fragments that differ by a single amino acid, NHCH(R)CO, and have the same number of charges generates a value that specifies the mass and thus the identity of the extra residue in the larger fragment (3, 16, 23). Residues 7–19 in the phosphopeptide were identified from the mass separation between fragments of type *y* that carry a single positive charge. Note that the mass difference between y_5 (m/z 689.3) and y_4 (m/z 446.3) is 243 Da, the expected value if residue 15 were phosphotyrosine. The remainder of the amino acid sequence in the phosphopeptide, residues 1–7, was determined from the mass separation between ions of type *y* that carry two positive charges. These are shown in the second row of masses below the structure in Fig. 1.

Ions of type *b* all contain the amino terminus plus 1, 2, 3, etc. additional residues (22). Subtraction of m/z values for any two fragments that differ by a single amino acid, NHCH(R)CO, and have the same number of charges generates a value that specifies the mass and thus the identity of the extra residue in the larger fragment (3, 16, 23). All but four of the assignments for residues 1–14 were confirmed by the presence of type *b* ions in the spectrum.

Analysis of $p42^{mapk}$ from *E. coli* After ^{32}P Labeling. As an additional approach to assess phosphorylation of $p42^{mapk}$ occurring *in situ*, bacteria containing the $p42^{mapk}$ plasmid were metabolically labeled with $^{32}P_i$ during induction, and the radiolabeled recombinant $p42^{mapk}$ was chromatographically purified (19). Aliquots of fractions from the final phenyl-Superose chromatography step were subjected to SDS/PAGE and autoradiography (data not shown). No incorporation of radiolabel into $p42^{mapk}$ eluted as form B was detected (data not shown). In contrast, $p42^{mapk}$ eluted as form A was radiolabeled (data not shown) and contained predominantly phosphotyrosine (Fig. 2), in general agreement with the analysis by mass spectrometry.

Identification of Tyr-185 as the *in Vitro* Phosphorylated Tyrosine in Recombinant $p42^{mapk}$ by Phosphopeptide Mapping and Mass Spectrometry. Form B of $p42^{mapk}$ undergoes an intramolecular autophosphorylation on tyrosine during *in vitro* phosphorylation (19). To determine whether this phosphorylation site is the same as the Tyr-185 that becomes phosphorylated in MAP kinase upon stimulation of EL4 cells with phorbol 12,13-dibutyrate (3), two-dimensional (high-voltage electrophoresis/thin-layer chromatography) tryptic phosphopeptide mapping studies were performed. A single phosphopeptide (peptide γ) was detected following autophosphorylation of form B of $p42^{mapk}$ *in vitro* with [γ-^{32}P]ATP (Fig. 3*A*). Only phosphotyrosine was detected in peptide γ by phospho amino acid analysis (data not shown).

For comparison, migrations of a synthetic peptide (VADPDHDHTGFLTEYVATR) phosphorylated on tyrosine by $pp60^{src}$ (peptide 1) and a doubly phosphorylated peptide (peptide 2) from a tryptic digest of ^{32}P-labeled MAP kinase isolated from phorbol ester-stimulated EL4 cells are shown (Fig. 3 *B* and *C*, respectively). The latter peptide contained phosphorylated Thr-183 and Tyr-185 (2). Peptide 1 can be resolved from peptide 2 by the two-dimensional peptide mapping procedure (Fig. 3*F*).

The unknown phosphopeptide, γ, comigrated with peptide 1 during high-voltage electrophoresis/thin-layer chromatography of a mixture of these two peptides (Fig. 3*E*). In addition, the map of a mixture of peptides γ and 2 (Fig. 3*D*)

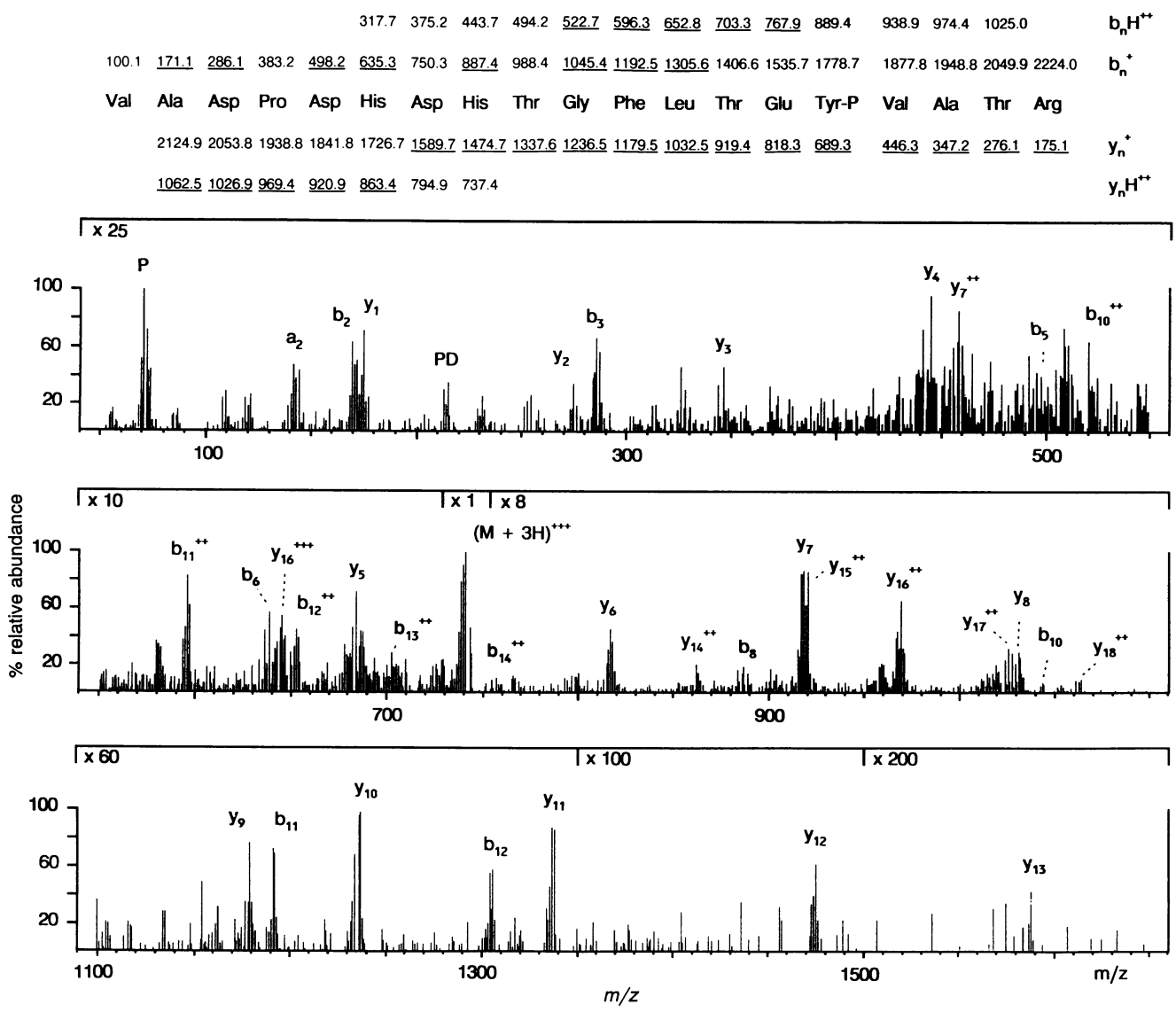

FIG. 1. CAD mass spectrum recorded on $(M + 3H)^{3+}$ ions (m/z 742, average mass) derived from the phosphorylated tryptic peptide of $p42^{mapk}$. Sample at the 5-pmol level was ionized under electrospray conditions. Predicted monoisotopic masses for fragments of types *b* and *y* from the deduced sequence are shown above and below the structure at the top of the figure. Those observed in the spectrum are underlined. Fragments that result from internal cleavage at proline are labeled with the appropriate single-letter codes to indicate the sequences contained in those fragments (22).

was identical to the map of a mixture of peptides 1 and 2 (Fig. 3*F*). Since the synthetic peptide contains only one tyrosine, Tyr-185, these data indicate that the *in vitro* phosphorylation of recombinant $p42^{mapk}$ also occurs on Tyr-185. In agreement with this conclusion, autophosphorylation of form B of $p42^{mapk}$ with $ATP/Mg^{2+}/Mn^{2+}$ resulted in a time-dependent increase in abundance of an $(M + 3H)^{3+}$ ion of m/z 742 (Fig. 4), identified by mass spectral analysis and phosphopeptide mapping as the tyrosine-phosphorylated regulatory peptide present in form A.

DISCUSSION

The structural studies described herein definitively establish the site of tyrosine phosphorylation of bacterially expressed $p42^{mapk}$ as Tyr-185, one of the two sites whose phosphorylation is required for full enzymatic activation. Mass spectrometry showed that Tyr-185 was essentially stoichiometrically phosphorylated in form A of $p42^{mapk}$ and was not phosphorylated in form B. These findings are consistent with the higher specific activity displayed by form A in comparison to form B (19). Moreover, tyrosine phosphorylation of $p42^{mapk}$ occurring during *in vitro* incubations of form B with [γ-^{32}P]ATP was also shown to occur on Tyr-185. The latter reaction is likely to be intramolecular because the rate of tyrosine phosphorylation of form B occurring *in vitro* is independent of protein concentration. Thus, recombinant $p42^{mapk}$, in the absence of any upstream factors, undergoes a slow, intramolecular phosphorylation on one of the two regulatory sites. This result implies that a similar reaction occurs for nonrecombinant $p42^{mapk}$. Autophosphorylation of murine $p42^{mapk}$ on tyrosine has been observed (19, 24). The extent to which this reaction might be accelerated in mammalian cells in response to growth factors, thus contributing to activation of $p42^{mapk}$ *in vivo*, is unknown. Recent work suggests that phosphorylation of Thr-183 and Tyr-185 may be catalyzed *in vivo* by an upstream MAP kinase kinase (25–27), but additional mechanisms have not been excluded.

The predominance of tyrosine phosphorylation of $p42^{mapk}$ occurring during expression of the protein in *E. coli* contrasts with the significant threonine phosphorylation that occurs during *in vitro* phosphorylations of purified forms A and B

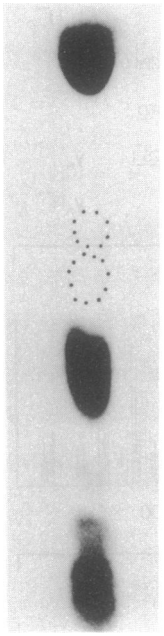

FIG. 2. Phospho amino acid analysis of $p42^{mapk}$ labeled *in situ*. A 50-ml culture of *E. coli* BL21(DE3)(pET-MK) was labeled with 10 mCi (370 MBq) of $^{32}P_i$ as described (19). $p42^{mapk}$ was purified as described (19). Fractions of $p42^{mapk}$ (form A) were pooled, concentrated, and electrophoresed in an SDS/10% polyacrylamide gel. ^{32}P-labeled $p42^{mapk}$ was excised and processed for phospho amino acid analysis. Migration of phospho amino acid standards is indicated. The origin is indicated by the arrowhead.

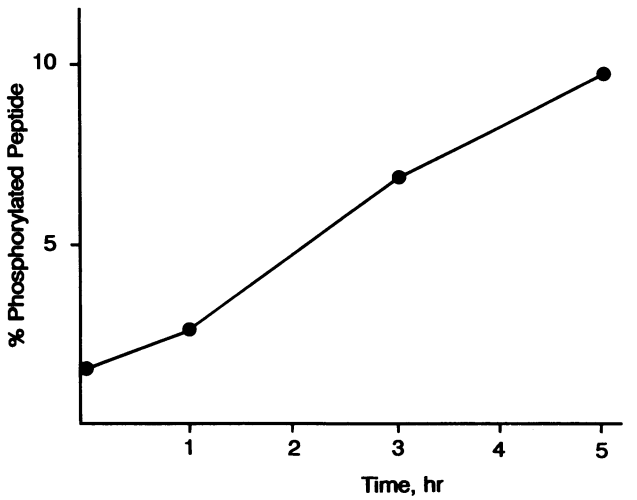

FIG. 4. Autophosphorylation of form B of $p42^{mapk}$. Aliquots of form B were autophosphorylated for the times indicated (abcissa), reactions were individually quenched by addition of EDTA, and material in each aliquot was digested with trypsin. The percentage of phosphorylated peptide in the mixture (ordinate) was calculated from the ratio of abundances observed for $(M + 3H)^{3+}$ ions (m/z 742) and $(M + 4H)^{4+}$ ions (m/z 557) derived from the phosphorylated and unphosphorylated forms, respectively.

during extended incubations (19). One plausible rationalization is based on the supposition, as yet unproven, that the observed threonine phosphorylation is intermolecular. Catalytic amounts of active MAP kinase will phosphorylate an excess of homogeneous MAP kinase that has been rendered inactive by heating (Timothy Haystead, personal communication). The tyrosine phosphorylation is intramolecular and is therefore not inhibited by competing *E. coli* protein substrates. Phosphorylation of substrate proteins on serine/threonine, however, is subject to substrate competition. When purified recombinant $p42^{mapk}$ is phosphorylated *in vitro*, no competing protein substrates are available. Under these conditions, both tyrosine and threonine phosphorylations of recombinant $p42^{mapk}$ are observed. The threonine sites have not been identified, beyond the exclusion of Thr-183 by peptide mapping (ref. 19 and this report).

How do dual phosphorylations at Thr-183 and Tyr-185 activate MAP kinase? Some kinases are inhibited basally by internal binding at the active site of a pseudosubstrate segment of amino acids, usually located at one or the other terminus, and are activated by relief of this inhibition. However, the sequence of MAP kinase (4) does not contain a segment at either terminus with a Pro-Xaa-Yaa-Pro motif (where Yaa is an amino acid other than Ser/Thr), the pseudosubstrate sequence predicted from the recognition

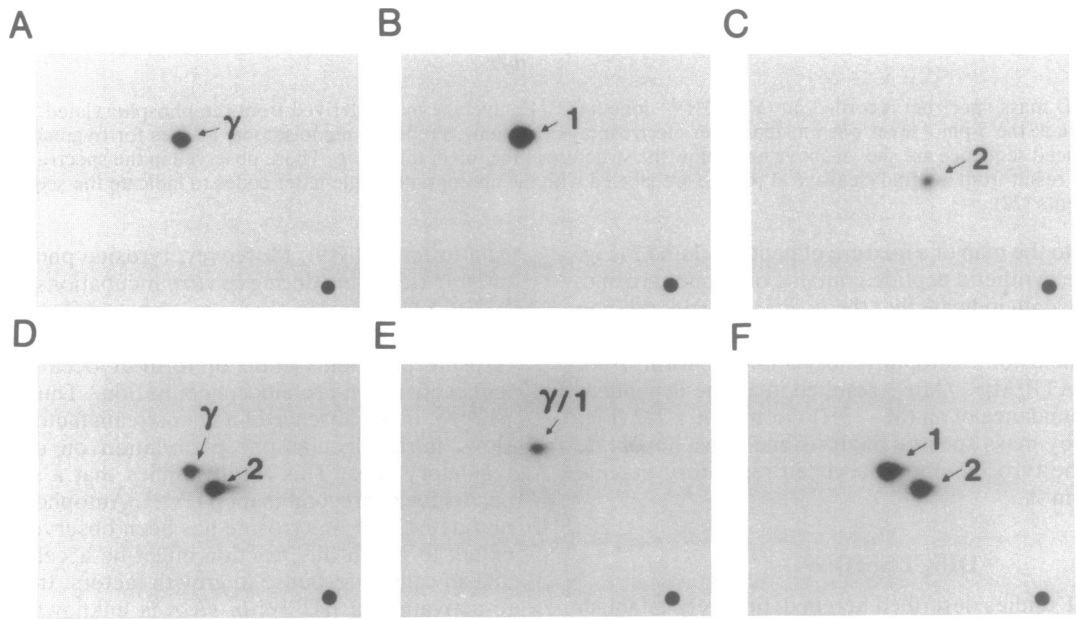

FIG. 3. Phosphopeptide map comparisons to identify the site of *in vitro* autophosphorylation in recombinant $p42^{mapk}$. The dot in the lower right-hand corner of each panel indicates the origin. Electrophoresis was towards the cathode (to left), followed by ascending chromatography. (*A*) Tryptic digest of recombinant $p42^{mapk}$ (form B) autophosphorylated *in vitro*. (*B*) Synthetic peptide, corresponding to the MAP kinase tryptic phosphopeptide, phosphorylated by $pp60^{src}$. (*C*) Tryptic digest of ^{32}P-labeled $p42^{mapk}$ isolated from phorbol 12,13-dibutyrate-treated EL4 cells. (*D*) Mixture of *A* and *C*. (*E*) Mixture of *A* and *B*. The phosphopeptide from recombinant $p42^{mapk}$ comigrates with the synthetic peptide phosphorylated by $pp60^{src}$. (*F*) Mixture of synthetic peptide phosphorylated by $pp60^{src}$ with the doubly phosphorylated MAP kinase phosphopeptide.

sequence proposed for the enzyme (28, 29). However, since some peptide substrates [e.g., Jun (7)] are phosphorylated by MAP kinase at sites that do not have Pro at the −2 position, the pseudosubstrate hypothesis cannot be categorically excluded.

Based on the crystal structure of the catalytic subunit of cAMP-dependent protein kinase (20), Tyr-185 (and Thr-183) in $p42^{mapk}$ are expected to be located in close proximity to conserved residues in the catalytic core. Thr-197 of the catalytic subunit of cAMP-dependent protein kinase is 9 residues amino-terminal from the conserved Ala-Pro-Glu in the amino acid sequence of this kinase. Thr-197 is phosphorylated, by a mechanism thought to be autophosphorylation, and once phosphorylated, Thr(*P*)-197 fulfills an important role in stabilization of the structure (20). Thr-197 is located in close proximity to the conserved catalytic fold, and thus Thr(*P*)-197 may be required for proper orientation of the catalytic residues. The phosphorylated residues in $p42^{mapk}$, Thr-183 and Tyr-185, are located 10 and 8 residues toward the amino terminus from the conserved Ala-Pro-Glu of $p42^{mapk}$. These considerations suggest that the tyrosine and threonine phosphorylations of members of the MAP kinase family may increase the enzymatic rate by proper orientation of the conserved residues responsible for catalysis. Modeling based on the structure of catalytic subunit of cAMP-dependent protein kinase may provide clues as to the conformational changes caused by the activating phosphorylations.

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