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The phorbol ester-dependent activator of the mitogen-activated protein kinase p42^{mapk} is a kinase with specificity for the threonine and tyrosine regulatory sites

(phosphatase 2A/casein kinase II/pp60^{src})

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ABSTRACT **Mitogen-activated protein kinases (MAP kinases) are activated by dual tyrosine and threonine phosphorylations in response to various stimuli, including phorbol esters. To define the mechanism of activation, recombinant wild-type 42-kDa MAP kinase (p42^{mapk}) and a kinase-defective mutant of p42^{mapk} (K52R) were used to assay both activator activity for p42^{mapk} and kinase activity toward K52R in stimulated EL4.I12 mouse thymoma cells. Phorbol 12,13-dibutyrate (10 min, 650 nM) stimulated a single peak of MAP kinase activator that was coeluted from Mono Q at pH 7.5 and 8.9 with K52R kinase activity. Both activities were inactivated by the serine/threonine-specific phosphatase 2A but not by the tyrosine-specific phosphatase CD45. Phosphorylation of K52R occurred specifically on Thr-183 and Tyr-185, as determined by tryptic phosphopeptide mapping in comparison with synthetic marker phosphopeptides. These findings indicate that phorbol ester-stimulated MAP kinase kinase can activate p42^{mapk} by threonine and tyrosine phosphorylations, and that p42^{mapk} thus does not require an autophosphorylation reaction.**

A prominent mechanism of signal transduction for growth factors, hormones, and neurotransmitters is activation of serine/threonine protein kinases. Among these, two closely related enzymes, p42^{mapk} and p44^{mapk}, are remarkable for the diversity of signals inducing their activation in different systems and for the mechanism of their activation by dual tyrosine and threonine phosphorylation (1, 2). The probable functions, differing nomenclature, and primary structure of the two mitogen-activated protein kinases (MAP kinases) and their relatives have been recently reviewed (3, 4). In quiescent cells capable of division, activation of MAP kinase correlates with reentry into the cell cycle.

To understand the regulation of MAP kinases, it is important to determine the nature and number of upstream factors participating in their activation. Phosphorylation of p42^{mapk} at both Thr-183 and Tyr-185 is required for full enzymatic activity toward protein substrates (5, 6). Removal of either phosphate by protein phosphatases decreases the enzymatic activity to <1% that of the doubly phosphorylated enzyme (5). The activating phosphorylations reside in the (partial) sequence FL$\overset{*}{T}$E$\overset{*}{Y}$VATRWYRAPE, 10 and 8 residues N-terminal from the conserved APE motif (underlined), a general location for autophosphorylations in other kinases (6).

Indeed, studies of recombinant p42^{mapk}/ERK2 and p44^{mapk}/ERK1 have revealed that both enzymes undergo slow endogenous phosphorylations on tyrosine and threonine (7–9). The tyrosine phosphorylation is intramolecular and is accompanied by weak activation of its enzymatic activity (8). These observations have been clarified by mass spectrometry and peptide mapping studies, which identified the site of intramolecular tyrosine phosphorylation as Tyr-185, the regulatory tyrosine site, and excluded Thr-183 as a site of significant phosphorylation in recombinant p42^{mapk} (ref. 8 and unpublished data). Endogenous phosphorylation and activation of MAP kinase also occur upon incubation of immunoprecipitates of p42^{mapk}/p44^{mapk} from mammalian cells together with ATP/Mg (10). However, coprecipitation of activating factor(s) cannot be excluded in this case. Thus, plausible mechanisms for activation include enhancement of autophosphorylation at one or both sites in addition to phosphorylation by a Thr-183 and/or Tyr-185 kinase(s), and combinations of these alternatives.

Previous studies have pointed to the existence and importance of an upstream protein activator but did not establish the mechanism of activation (11–13). An activating factor(s) was demonstrated by Ahn *et al.* (11) in fibroblasts treated with epidermal growth factor (EGF) or phorbol esters, with *in vitro* activation of partially purified mammalian MAP kinase as an assay, and its size was estimated as 50–60 kDa by gel filtration. MAP kinase activator has also been demonstrated in phorbol ester-treated fibroblasts (11) and UM937 cells (12). MAP kinase activator was partially purified from nerve growth factor (NGF)-treated PC12 cells by Gómez and Cohen (13). The NGF-dependent activator was inactivated by phosphatase 2A, but not CD45, suggesting that the activator itself is regulated by serine/threonine phosphorylation (13). Its abilities to induce both threonine and tyrosine phosphorylation of MAP kinase were inactivated in parallel by treatment with phosphatase 2A, suggesting that both were catalyzed by the same protein (13). Finally, strong evidence for a MAP kinase kinase (MAPKK) has been provided in *Xenopus laevis* by the demonstration that kinase-defective mutants of *Xenopus* MAP kinase injected into oocytes are phosphorylated on threonine and tyrosine and that mutants with nonphosphorylatable substitutions at sites corresponding to Thr-183 and Tyr-185 are not (14).

To define the nature of the MAP kinase activator and provide details of the mechanism in mammalian cells, we have studied in parallel the ability of Mono Q fractions from phorbol 12,13-dibutyrate (PBt_2)-stimulated EL4 cells to reactivate recombinant p42^{mapk} enzymatic activity and phosphorylate the Lys-52 → Arg (K52R) mutant of p42^{mapk}, which lacks enzymatic activity. Using synthetic tryptic peptide phosphorylated on Thr-183 and/or Tyr-185 prepared *in vitro* as markers, we demonstrate that the activating factor in PBt_2-stimulated thymocytes possesses kinase activity specific for Thr-183 and Tyr-185.

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Abbreviations: NGF, nerve growth factor; EGF, epidermal growth factor; MAP kinase, mitogen-activated protein kinase; MBP, myelin basic protein; MAPKK, MAP kinase kinase; PBt_2, phorbol 12,13-dibutyrate; HVE/TLC, high-voltage electrophoresis/thin-layer chromatography; CK II, casein kinase II.

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MATERIALS AND METHODS

Materials. Recombinant $p42^{mapk}$ and the K52R mutant were purified as described (8). Synthetic tryptic peptide containing the regulatory phosphorylation sites (asterisks), VADPAHDHTGFL*T*EY*VATR, was purchased from Synthecell (Rockville, MD). Purified $pp60^{src}$, phosphatase 2A, CD45, and casein kinase II (CK II) were generous gifts of Nick Lydon (CIBA–Geigy), Tim Haystead (University of Virginia), Nick Tonks (Cold Spring Harbor Laboratory), and Claiborne Glover III (University of Georgia), respectively.

Preparation of Lysate Supernatants and Mono Q Chromatography. Cellular supernatants of PBt_2-stimulated (650 nM, 10 min) or control (unstimulated) EL4.IL2 mouse thymoma cells were prepared and stored at −70°C exactly as described (6). Prior to application, thawed aliquots of supernatant from 6×10^8 cells (1–1.4 mg of protein per ml) were passed through 0.2-μm filters.

Analytical Mono Q chromatography at pH 7.5 was performed essentially as described (2) with buffer A (2) and buffer A containing 240 mM NaCl (total) as equilibration and elution buffers, respectively. For Mono Q chromatography at pH 8.9, fractions containing MAPKK activity from chromatography at pH 7.5 (above) were pooled and adjusted to pH 8.9 just prior to application to columns equilibrated in pH 8.9 buffer A. In this instance, the column was washed with 6 ml of pH 8.9 equilibration buffer followed by elution with a 12-ml NaCl gradient (0.025 M–1 M) in 0.5-ml fractions.

Assay for Activating Factors for $p42^{mapk}$. Sample (10 μl) was incubated at 30°C for 40 min with or without 180 ng of homogeneous $p42^{mapk}$ in 40 μl (total volume) of 25 mM Hepes, pH 7.5/1 mM dithiothreitol/10 mM $MgCl_2$/50 μM [γ-^{32}P]ATP (7 cpm/fmol) with myelin basic protein (MBP) at 0.5 mg/ml. The reaction was terminated by spotting 38 μl of each reaction mixture onto (1 cm^2) P81 cellulose paper (Whatman) and immersing the filter in 180 mM H_3PO_4. Thereafter, filters were washed (15) and retained radioactivity was quantitated by measurement of Cerenkov radiation. Incorporated radioactivity, corrected for background, was expressed in pmol/min.

Assay for MAPKK Phosphotransferase Activity. Sample (10 μl) was incubated at 30°C for 25 min with 0.8 μg of the K52R mutant of $p42^{mapk}$ (8) in 40 μl (total volume) of 10 mM Hepes, pH 7.5/40 μM [γ-^{32}P]ATP (5 cpm/fmol)/15 mM $MgCl_2$/1 mM dithiothreitol. The reaction was stopped by the addition of 5× Laemmli sample buffer. After SDS/10% PAGE, incorporation of $^{32}P_i$ into K52R was quantitated by autoradiography and/or Cerenkov radioactivity of excised bands and expressed in pmol/min.

Concentration and Desalting of MAPKK for Phosphatase Treatment. Fractions containing the MAPKK activity from sequential Mono Q chromatographies at pH 7.5 and 8.9 were pooled (1 ml). The buffer was exchanged by using a Sephadex G-25 fastflow desalting column (Pharmacia LKB) equilibrated with 20 mM Hepes, pH 7.5/10% (vol/vol) glycerol/10 mM NaCl/1 mM dithiothreitol and was concentrated to ≈100 μl with a Centricon 10 (Amicon). Aliquots (2 μl) were treated with or without phosphatases for 1 hr essentially as described (5), but with 1 μM okadaic acid instead of fluoride/EDTA for inactivation of phosphatase 2A.

Preparation of Thr-183- and/or Tyr-185-Phosphorylated Regulatory Tryptic Peptides. Synthetic peptide (23 nmol, 50-μl total volume) was phosphorylated at 30°C with homogeneous $pp60^{src}$ (0.3–0.4 μg), for either 80 min with 60 μM [γ-^{32}P]ATP (5 cpm/fmol) or for 15–18 hr with 1 mM ATP in 20 mM Hepes, pH 7.5/2 mM Na_3VO_4/1 mM dithiothreitol/10 mM $Mg(OAc)_2$. The reaction was stopped with formic acid (10% vol/vol, final).

Samples were diluted 2-fold with water and applied to a C_{18} column (25 × 0.46 cm, VYDAC, Hesperia, CA). Solvent A was 0.1% (vol/vol) trifluoroacetic acid in water; solvent B was 0.08% trifluoroacetic acid in acetonitrile/water (80:20). The column was washed with 5 ml of solvent A, followed by elution at 0.5 ml/min by a gradient with linear segments (0–40% B and 40–50% B, 15 min each) in 0.5-ml fractions. Under these conditions, Tyr ([^{32}P]*P*) peptide (2–3 × 10^6 Cerenkov cpm) was resolved from the parent peptide and eluted at 38–41% acetonitrile. The peak of Tyr(*P*) peptide from overnight phosphorylation with nonradioactive ATP was dried by a Speedvac (Savant) for phosphorylation with CK II.

To prepare marker peptide doubly phosphorylated on threonine and tyrosine, nonradioactive Tyr(*P*) peptide (9–14 nmol) was phosphorylated (30°C, 2–3 hr) with homogeneous CK II (0.25 μg) and [γ-^{32}P]ATP/Mg, under similar buffer conditions as above but including 0.1 M NaCl (total). Singly phosphorylated peptide radiolabeled on Thr-183 was prepared by phosphorylation of unphosphorylated peptide with CK II under similar conditions. The products were separately chromatographed, and the [^{32}P]phosphopeptides were identified from Cerenkov radioactivity. Typically, 0.3–1 × 10^6 Cerenkov cpm of Thr([^{32}P]*P*) peptide and Thr([^{32}P]*P*)/Tyr(*P*) peptide were obtained, which did not resolve from their respective nonradioactive precursors.

Assignment of phosphorylation at Thr-183 and Tyr-185 was made by mass spectrometry and phospho amino acid analysis (unpublished data); [^{32}P]phosphopeptides were stored in eluant at −70°C and aliquots were dried before use.

Other Methods. Procedures for phospho amino acid analysis and two-dimensional tryptic phosphopeptide mapping were described (2).

RESULTS

Activation of Recombinant $p42^{mapk}$ by a Phorbol Ester-Induced Activator. Assays for activation of $p42^{mapk}$ were performed on aliquots of chromatographic fractions from Mono Q chromatography (pH 7.5) of lysates of PBt_2-stimulated and unstimulated thymoma cells (Fig. 1*A*). Each fraction was assayed for phosphotransferase activity toward MBP with and without inclusion of homogeneous, unphosphorylated B form of recombinant $p42^{mapk}$ (8) in the reaction mixture. The added unphosphorylated MAP kinase makes a negligible contribution to the measured MBP kinase activity. In assays performed in the absence of recombinant $p42^{mapk}$, two peaks of MBP kinase activity (fractions 32–38; fractions 40–48) were detected in fractions from PBt_2-treated cells but were essentially absent in cells left unstimulated. These peaks result from activation of endogenous $p42^{mapk}$ and $p44^{mapk}$, as expected (2, 11). However, assays including the added recombinant protein revealed a peak of MBP kinase activity due to the activator activity. The activator activity (fractions 1–10) was eluted in the wash fractions and was absent in unstimulated cells. These data validate use of the recombinant protein to assay MAP kinase activation factors. This assay will be invaluable for purification of sufficient protein for structural analysis and cloning.

Since the MAP kinase activator did not bind to Mono Q at pH 7.5 (Fig. 1*A*), the active fractions were reapplied at a higher pH. Pooled fractions 1–10 from PBt_2-treated cells were adjusted to pH 8.9 and reapplied to Mono Q equilibrated at that pH. The activator bound and was eluted as a single peak at ≈0.3 M NaCl (Fig. 2*A*), indicative of a pI between 7.5 and 8.9.

Phosphorylation of the K52R mutant of $p42^{mapk}$ by MAPKK. Kinase assays to measure phosphotransferase activity toward the K52R mutant of $p42^{mapk}$ as substrate were performed in parallel, using the same chromatographic fractions assayed for activating factor(s) in Figs. 1*A* and 2*A*. Fractions containing activator phosphorylated the K52R mutant pro-

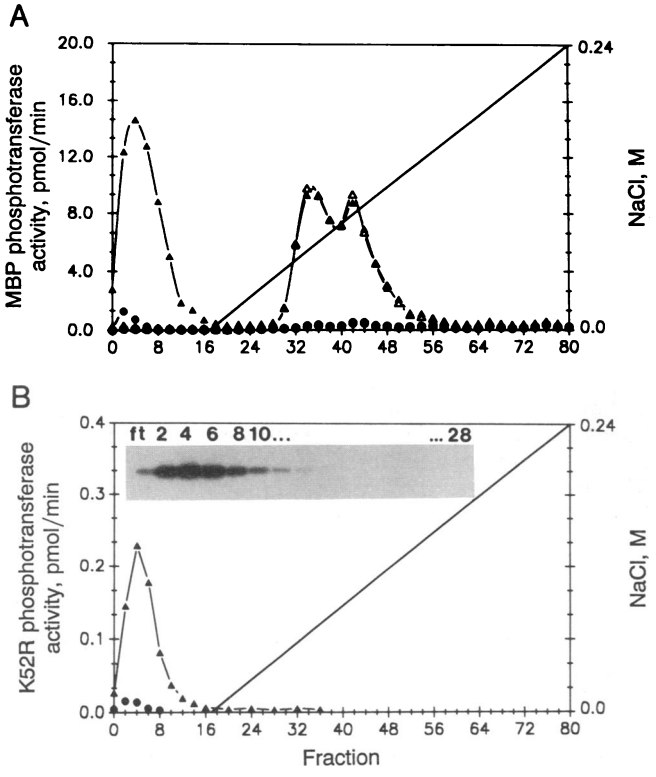

FIG. 1. Coelution of MAP kinase activator and K52R phosphotransferase activities from Mono Q at pH 7.5. (*A*) Fractions were assayed for ability to stimulate the MBP phosphotransferase activity (left ordinate) of recombinant $p42^{mapk}$. Triangles, PBt_2-stimulated cellular fractions with (▲) and without (△) recombinant $p42^{mapk}$; circles, unstimulated cellular fractions with (●) and without (○) recombinant $p42^{mapk}$. The unphosphorylated form B $p42^{mapk}$ (8) was used as substrate. (*B*) K52R phosphotransferase activity of PBt_2-stimulated (▲) and unstimulated (●) fractions from *A*. (*Inset*) Autoradiograph of K52R from assays of indicated fractions, PBt_2-stimulated cells. ft, Flowthrough. Line in each panel shows predicted gradient (right ordinate).

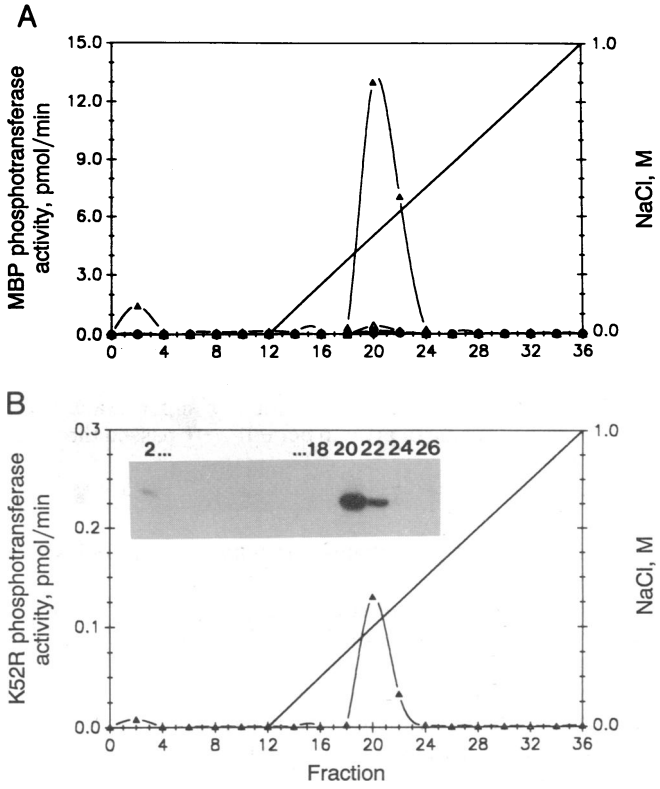

FIG. 2. Comigration of MAP kinase activator and K52R phosphotransferase activities from Mono Q at pH 8.9. Fractions containing MAP kinase activator from chromatography at pH 7.5 (Fig. 1*A*) were rechromatographed at pH 8.9. (*A*) MAP kinase activator, symbols as in Fig. 1*A*. (*B*) K52R phosphotransferase activity (left ordinate) of PBt_2-stimulated cells. (*Inset*) As in Fig. 1*B*.

tein (Figs. 1*B* and 2*B*), and the two activities were proportional. Fractions having the highest activity as an activator also had the highest MAPKK activity. In addition, activation of MAPKK activity was similarly dependent on stimulation by phorbol ester (Fig. 1*B*).

Substitution of arginine for the conserved active-site lysine in the K52R mutant renders the protein kinase-defective. The K52R mutant does not autophosphorylate (8) and does not phosphorylate MBP (data not shown) even when incubated with the activating fractions under conditions that result in phosphorylation of Thr-183 and Tyr-185 (see below). Thus, the observed correspondence between phosphorylation of K52R and activation of $p42^{mapk}$ strongly implies that the activating factor is a MAPKK, as suggested by Gómez and Cohen (13).

Inactivation of Both Activator and MAPKK Activities by Phosphatase 2A. Gómez and Cohen (13) have shown that protein phosphatase 2A, but not CD45, inactivates the MAP kinase-activating factor stimulated by NGF in PC12 cells. For this reason, we studied the ability of phosphatase 2A to inactivate the K52R phosphotransferase activity present in fractions containing the activator (Fig. 3*A*), as well as the activity that stimulates MBP kinase activity of recombinant $p42^{mapk}$ (Fig. 3*B*).

Incubation of K52R with [γ-^{32}P]ATP/Mg and MAPKK, purified by the sequential Mono Q chromatographies described in Figs. 1 and 2, resulted in phosphorylation of K52R (Fig. 3*A*, lane UT). Phosphorylation of K52R was abolished when an equivalent aliquot was treated with phosphatase 2A (lane 2A). In contrast, phosphorylation of K52R was not inhibited by incubation with CD45 (lane CD). Addition of okadaic acid (1 μM) to the incubation with phosphatase 2A blocked inactivation of K52R phosphotransferase activity (lane 2A OA), indicating that the observed inactivation was

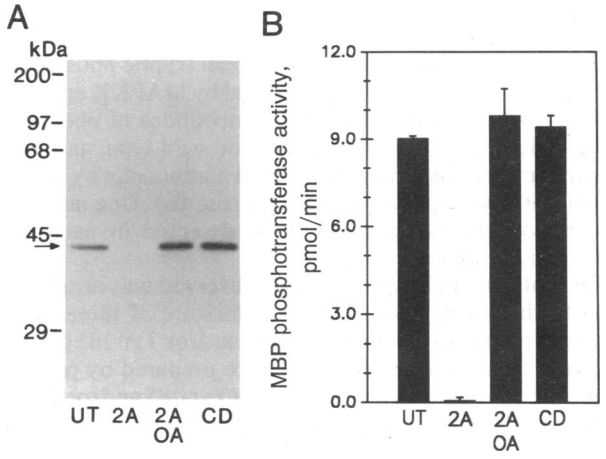

FIG. 3. Inactivation by protein phosphatase 2A of both K52R phosphotransferase and MAP kinase activator activities present in a concentrate of pooled fractions from sequential pH 7.5 and pH 8.9 Mono Q chromatographies. (*A*) Autoradiograph of K52R (arrow) phosphorylated by MAPKK that was untreated (UT), treated with phosphatase 2A (2A), treated with phosphatase 2A in the presence of okadaic acid (2A/OA), or treated with CD45 (CD). (*B*) MBP phosphotransferase activity of aliquots NT, 2A, 2A/OA, and CD. Average of duplicate assays is shown with the range of the values (bar).

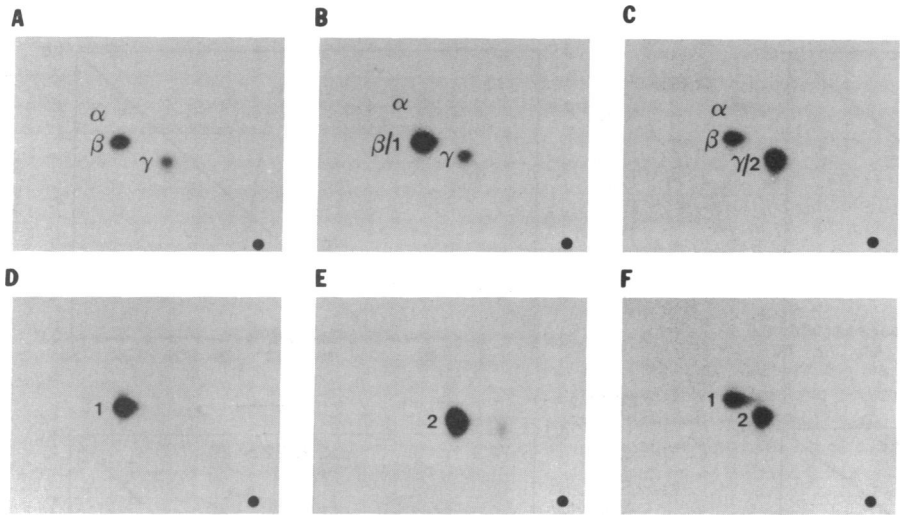

FIG. 4. Identification of Thr-183 and Tyr-185 as the sites phosphorylated by MAPKK. Phosphorylated K52R eluted from an SDS/10% polyacrylamide gel (Fig. 1*B Inset*, pool of lanes 2, 4, 6, and 8) was subjected to trypsin proteolysis followed by high-voltage electrophoresis/thin-layer chromatography (HVE/TLC). (*A*) Phosphopeptides identified in K52R (labeled α, β, and γ). (*B*) Mixture of phosphopeptides from *A* and *D*. (*C*) Mixture of phosphopeptides from panels *A* and *E*. (*D*) Tyrosine-phosphorylated synthetic peptide (labeled 1). (*E*) Doubly phosphorylated synthetic peptide (labeled 2). (*F*) Mixture of phosphopeptides from *D* and *E*. Dot in the right-hand corner represents the origin.

a consequence of phosphatase activity, not proteolysis or thermal denaturation.

Similar effects of incubation with phosphatase 2A on the ability of the fraction to stimulate $p42^{mapk}$ enzymatic activity were observed (Fig. 3*B*). Treatment with phosphatase 2A reduced activation of $p42^{mapk}$ by 95–100%, relative to control reactions, and no reduction occurred in the presence of okadaic acid. Furthermore, treatment with CD45 did not diminish activation of $p42^{mapk}$.

Thus, both phosphorylation of the K52R mutant (Fig. 3*A*) and stimulation of enzymatic activity of recombinant $p42^{mapk}$ (Fig. 3*B*) are inactivated by 2A, but not CD45. Inactivation of both activities by phosphatase 2A, but not CD45, supports the conclusion that the MAPKK is the activator.

Site Identification by Phosphopeptide Mapping and Phospho Amino Acid Analysis. Equal quantities (0.8 μg) of recombinant $p42^{mapk}$ and K52R proteins were separately phosphorylated *in vitro* by MAPKK [peak fractions from Mono Q (pH 7.5) chromatography of lysates of PBt_2-treated cells]. In multiple experiments, incorporations of radiolabel into wild-type and kinase-defective mutants, assessed after SDS/PAGE, were nearly equal (data not shown), consistent with autophosphorylation being quantitatively negligible in comparison to incorporation from the kinase reaction.

Typical results from two-dimensional tryptic phosphopeptide mapping of K52R phosphorylated by MAPKK are shown in Fig. 4*A*. The patterns of phosphopeptides of phosphorylated wild-type MAP kinase and of wild-type and K52R phosphorylated by activator purified additionally by Mono Q at pH 8.9 were equivalent (data not shown). One minor and two major phosphopeptides were detected in each case, labeled α, β, and γ.

The pattern of phosphopeptides observed was nearly identical to that from HVE/TLC of a mixture of three marker peptides phosphorylated on Thr-183 and/or Tyr-185 (data not shown). These marker peptides were prepared by phosphorylating a synthetic tryptic peptide on Tyr-185 and/or Thr-183 with $pp60^{src}$ and CK II, used separately or sequentially (see *Materials and Methods*). $pp60^{src}$ is specific for tyrosine residues, whereas CK II requires acidic residues one to three residues C-terminal to acceptor serine/threonine (16) and preferentially phosphorylates Thr-183. The doubly phosphorylated marker comigrated with regulatory [^{32}P]phosphopeptide from $p42^{mapk}$ from EL4.I12 cells labeled *in situ* and stimulated with PBt_2 (unpublished data). The similarity in mobilities suggested that peptides α, β, and γ are the threonine-, tyrosine-, and doubly phosphorylated regulatory peptides, respectively. This supposition was confirmed by detailed comparison of phosphopeptide mobilities (Fig. 4 *B–F*).

Mobilities of synthetic Tyr([^{32}P]*P*)- and Thr([^{32}P]*P*)/Tyr(*P*)-containing marker phosphopeptides during HVE/TLC are shown individually in Fig. 4 *D* and *E*, respectively, and as a mixture of the two in Fig. 4*F*. When Tyr([^{32}P]*P*) peptide was mixed with the tryptic digest of phosphorylated K52R (Fig. 4*A*), the intensity of peptide β increased (Fig. 4*B*). Similarly, when Thr([^{32}P]*P*)/Tyr(*P*) peptide was mixed, the intensity of peptide γ increased (Fig. 4*C*). Therefore, peptides β and γ are the regulatory tryptic peptide singly phosphorylated on Tyr-185 and doubly phosphorylated on Thr-183 and Tyr-185, respectively.

Peptide β contained only phosphotyrosine (Fig. 5, lane β), whereas peptide γ contained approximately equal amounts of phosphotyrosine and phosphothreonine (lane γ), consistent with the identification of peptides β and γ given above. Phospho amino acid analysis of marker phosphopeptide singly phosphorylated on Tyr-185 detected only phosphotyrosine (lane 1), demonstrating that contaminating serine/threonine kinases were not present in the $pp60^{src}$ used in preparing these markers. Marker Thr([^{32}P]*P*)/Tyr(*P*) peptide contained phosphothreonine as the only detectable

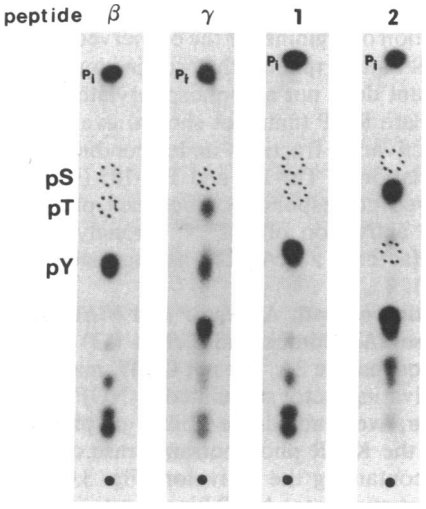

FIG. 5. Phospho amino acid analysis of the phosphopeptides identified in Fig. 4. Lanes contained phosphopeptides β, γ, 1, and 2, respectively. Positions of the phospho amino acid standards (pS, phosphoserine; pT, phosphothreonine; pY, phosphotyrosine) (circled by small dots) and free P_i are indicated.

[^{32}P]phospho amino acid (lane 2), consistent with its preparation from a nonradioactive Tyr(*P*) peptide.

DISCUSSION

The mechanism of activation of MAP kinase in response to phorbol esters has been particularly perplexing. Phorbol esters stimulate the same regulatory threonine and tyrosine phosphorylations of p42^{mapk} as are induced by peptide mitogens whose receptors are tyrosine kinases (6, 12, 15), whereas the "receptor" for phorbol esters, protein kinase C, is specific for serine/threonine residues. The principal ambiguity in our understanding was removed by the demonstration that one or more upstream activators of MAP kinase are stimulated by EGF and NGF and (presumptively) regulated by serine/threonine phosphorylation (11, 13).

The data herein establish that activation of MAP kinase in response to phorbol esters occurs by activation of a MAPKK activity with specificity for both the threonine and tyrosine regulatory sites. The PBt_2-stimulated activator copurifies during Mono Q chromatographies with phosphotransferase activity toward K52R, a kinase-defective p42^{mapk}, excluding contributions from autophosphorylation in this case. Activator and MAPKK also copurify during cation-exchange and gel filtration chromatographies (unpublished data). Both phosphotransferase activity toward K52R and activation of p42^{mapk} are abrogated by treatment with phosphatase 2A, and not by CD45. This finding is consistent with the demonstration by Gómez and Cohen (13) that phosphatase 2A inactivated both apparent threonine and apparent tyrosine kinase activities of a NGF-stimulated MAPKK using mammalian p42^{mapk} inactivated with phosphatase 2A or with CD45, respectively, as substrate. Copurification of activator activity with K52R phosphotransferase activity, and inactivation of both by phosphatase 2A, strongly implies that the activator is a kinase.

Phosphopeptide mapping demonstrated that MAPKK phosphorylates the regulatory sites, Thr-183 and Tyr-185, in K52R. Identification of Tyr-185 as a site is unequivocal since the regulatory tryptic peptide contains only one tyrosine. Although the threonine-phosphorylated markers are phosphorylated on Thr-183, identification of Thr-183 as a site based on phosphopeptide mapping strictly requires that an additional argument be appended as follows. Fully active p42^{mapk} from phorbol ester-treated cells is phosphorylated on Thr-183 and Tyr-185 in the cells (6). The observed MBP kinase activities resulting from phosphorylation of recombinant p42^{mapk} with MAPKK are large, consistent with generation of the fully active enzyme. Therefore, comigration of a phosphopeptide from K52R with a marker peptide known to be doubly phosphorylated on Thr-183 and Tyr-185 strongly implies that the threonine residue phosphorylated by MAPKK is Thr-183.

The phosphopeptide mapping data suggest that the order of phosphorylation of Thr-183 and Tyr-185 may not be strictly random. Comparatively little phosphopeptide corresponding to Thr-183 phosphorylation (peptide α) was observed in tryptic digests of phosphorylated p42^{mapk}, relative to tyrosine-phosphorylated and doubly phosphorylated Tyr(*P*)/Thr(*P*) peptide.

MAPKK appears to be very specific for MAP kinase as substrate. A broad examination of proteins conventionally used as kinase substrates has failed to identify a useful conventional kinase substrate protein for MAPKK (11). We find that Mono Q fractions containing active MAPKK will phosphorylate, albeit poorly, the synthetic regulatory peptide (1 mg/ml), generating phosphopeptides that comigrate during HVE/TLC with marker peptides phosphorylated singly on tyrosine or threonine (unpublished data).

Presently, the MAP kinase activator appears to be a single MAPKK protein or protein complex with dual specificity for the Thr-183 and Tyr-185 regulatory sites. Reactivating activities for MAP kinase deactivated by either tyrosine or threonine dephosphorylation comigrated during Mono Q and gel filtration chromatographies of the EGF-dependent activator from fibroblasts (11). Similarly, both activities from NGF-stimulated PC12 cells comigrated during Mono Q, Mono S, and Superose 12 chromatographies and were inactivated in parallel by phosphatase 2A (13). The activator from insulin-stimulated skeletal muscle has been purified ≈3000 fold, with preservation of tyrosine and threonine kinase activity (S. Nakielny and P. Cohen, personal communication). These observations and our data herein suggest that MAP kinase activator is a single protein (or protein complex) of 40–60 kDa that binds weakly (or not at all) to Mono Q at pH 7.5 and is inactivated by phosphatase 2A but not CD45. Assignment of both Thr-183 and Tyr-185 kinase activities to one protein will remain tentative until MAPKK is purified for sequencing and cloned.

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