Transcription Factor Phosphorylation by pp90<sup>rsk2</sup>

IDENTIFICATION OF Fos KINASE AND NGFI-B KINASE I AS pp90<sup>rsk2</sup>

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The in vitro phosphorylation of transcription factors by growth factor-activated protein kinases has resulted in the discovery of a number of activities whose identities and relationships to one another are unclear. Fos kinase is a growth factor-stimulated serine/threonine protein kinase that phosphorylates c-Fos at serine 362 within the carboxyl-terminal regulatory domain. Fos kinase activation is dependent on p21<sup>ras</sup> and mitogen-activated protein kinase/ERK kinase kinase (MEK) activity and is independent of phosphatidylinositol 3-kinase activity. We have purified Fos kinase by affinity chromatography using the Sepharose-linked protein kinase inhibitor, bisindolylmaleimide (BIM). Fos kinase has an apparent molecular mass of 88 kDa, and mass spectrophotometric analysis of the isolated protein showed that it produced tryptic fragments identical to those predicted for pp90<sup>rsk2</sup>. Fos kinase isolated from nerve growth factor-stimulated PC12 cells is indistinguishable from NGFI-B kinase I, based on their chromatographic behavior, substrate specificities, and relative sensitivity to BIM. Furthermore, we have distinguished Fos kinase from calcium/cAMP response element-binding protein (CREB) kinase. Therefore, Fos kinase and NGFI-B kinase I and pp90<sup>rsk2</sup> represent the same protein kinase species. Moreover, we report that pp90<sup>rsk2</sup> exists within nerve growth factor-stimulated PC12 cells as two chromatographically and immunologically distinct species. Finally, we demonstrate that CREB kinase is distinct from pp90<sup>rsk2</sup>.

Cells respond to various extracellular stimuli including growth factors, hormones, and changes in environmental conditions through the activation of intracellular signal transduction networks. Investigations of such signaling pathways have largely focused on the identification of constituents of these pathways with the aim of modeling functional cascades that lead from activated receptors to specific cytoplasmic and nuclear effectors (1, 2). The activity of many enzymes that comprise these cascades is regulated by their phosphorylation state. Thus, protein kinases and phosphatases play central roles in the sequential transfer of information through such cascades. We previously identified a growth factor-inducible serine/threonine protein kinase, termed Fos kinase, whose activity is rapidly induced after growth factor stimulation (3). Fos kinase was identified and so named by its in vitro ability to phosphorylate serine 362 of the proto-oncogene product, c-Fos, as well as a synthetic peptide whose sequence consists of 12 carboxyl-terminal amino acids of the c-Fos protein (4). Blenis and colleagues (5) demonstrated that serines 362 and 374 were in vitro substrates for pp90<sup>rsk2</sup> and ERK, respectively. Mutation of these serines to glutamate residues resulted in the increased ability to transform NIH 3T3 cells that was correlated with increased protein half-life, suggesting that phosphorylation at these sites within c-Fos promoted its stability and thus elevated AP-1 levels (6, 7). These data suggested that Fos kinase activity may be involved in promoting growth factor-induced changes in transcription (4).

Protein kinases are typically identified through their ability to phosphorylate recombinant proteins or synthetic peptides in vitro. This convention results in the confusion of different enzymes due to their relaxed substrate specificity arising from promiscuous phosphorylation of substrates. One goal of the present study was to establish the identities and relationships of protein kinases that phosphorylate similar sites within the transcription factors c-Fos, CREB, and NGFI-B.

NGFI-B kinase I was identified in PC12 cells as a growth factor-stimulated protein kinase able to phosphorylate the orphan steroid receptor NGFI-B (nur77), an immediate early gene product (8, 9). Phosphorylation of NGFI-B at serine 350 results in the loss of its DNA-binding ability. NGFI-B is expressed following both depolarization and growth factor stimulation. However, NGFI-B is only phosphorylated by NGFI-B kinase I only in response to growth factor stimulation, reflecting the complexity of the NGFI-B regulation. When NGFI-B is expressed as a result of growth factor stimulation, it was predominantly found in the cytoplasm, whereas when its expression was driven by membrane depolarization it was located within both the nucleus and cytoplasm (10). This suggests a role for NGFI-B kinase I in controlling the cellular distribution of this protein as well as governing its ability to bind its DNA element. NGFI-B kinase I shares many characteristics with Fos kinase including similar behavior on chromatographic resins, substrate recognition sequences, and activation kinetics downstream of growth factor stimulation (4, 8) and prompted...
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us to compare directly these protein kinases to determine their relationship to each other.

The transcription factor, calcium/cAMP response element binding protein (CREB), is constitutively bound to the calcium response element (CRE). Phosphorylation of CREB at serine 133 confers its ability to activate transcription from the CRE (11). This site has been shown to be phosphorylated by protein kinase A, PKC, and calmodulin kinase II and MAP kinase-activated protein kinase 2 (12). Protein kinases that function within signal transduction pathways mediate the action of a variety of different stimuli. Another enzyme capable of phoshorylating this site, CREB kinase, was described by Xing et al. (13) and subsequently identified as a growth factor-stimulated member of the 90-kDa ribosomal S6 kinase family, pp90^ras^2. Like pp90^ras^2, CREB kinase activation is dependent on p21^ras^ (14) and functions as a downstream element of the MAP kinase cascade. p90^ras^ family members act to effect changes in gene expression through the phosphorylation and activation of nuclear transcription factors including CREB (13) and c-Fos (6). CREB kinase and NGFI-B kinase I exhibit similar biochemical features, including their behavior on Mono Q columns and substrate specificities, leading us to question whether Fos kinase, NGFI-B kinase I, and/or CREB kinase represented the same enzymatic species. We report that Fos kinase and NGFI-B kinase I represent a single protein kinase species that is indistinguishable from pp90^ras^2. However, our findings demonstrate that CREB kinase and Fos kinase represent different species, casting doubt on the identity of CREB kinase as pp90^ras^2 (13). A primary finding of this study was that pp90^ras^2 exists within PC12 cells in two chromatographically and immunologically distinct species.

MATERIALS AND METHODS

Tissue Culture—PC12 cells were maintained in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) supplemented with 10% horse serum and 5% fetal bovine serum (Intergen, Purchase, NY) in an atmosphere of 10% CO_2 at 37 °C. PC12 cells expressing a dominant negative form of p21^ras^ (GRKrasDN6) cells (15) were incubated with 2 μM dexamethasone for 24 h to induce expression of the transgene before stimulation with NGF and hCG.

Chromatographic Characterization—PC12 cells were harvested by gentle trituration from tissue culture plates, suspended in phosphate-buffered saline, pH 7.4, containing 1 mg/ml bovine serum albumin and 1 mg/ml glucose and stimulated as indicated. The cells were collected by centrifugation and lysed by sonication in TEV buffer (20 mM Tris, pH 7.4, 1 mM EDTA, and 100 μM orthovanadate) containing 20 mM p-nitrophenyl phosphate, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml aprotinin. The lysates were centrifuged at 100,000 × g for 30 min, and the supernatants were applied to a Mono Q HR 5/5 column (Amersham Pharmacia Biotech) previously equilibrated in TEV. The column was developed at a flow rate of 1 ml/min and developed with a 40-ml 0–0.5 M NaCl linear gradient, and 1-ml fractions were collected. All procedures were carried out at 4 °C. Protein concentrations were determined by the method of Bradford (16).

Determination of Protein Kinase Activity—Fos kinase activity was assayed by the phosphorylation of a peptide substrate encompassing the serine 362 phosphorylation site (RKGSSSNEPSSD; 250 μM) in the presence of 20 mM Tris, pH 7.4, 10 μM [γ-32P]ATP (22 dpm/nmol), 10 mM MgCl_2, and 5 mM p-nitrophenyl phosphate. Reactions were carried out in a final volume of 50 μl and terminated after 20 min at room temperature by the addition of 10 μg of bovine serum albumin and trichloroacetic acid to a final concentration of 3.5%. After 15 min at 4 °C, the samples were centrifuged to remove precipitated proteins, and 25 μl of the resulting supernatants were spotted in triplicate onto P81 paper (Whatman) (17). The P81 papers were then washed 4 times for 5 min each in 75 mm phosphate buffer to remove unincorporated radioactivity. Incubation was determined by Cerenkov counting of the washed papers. Assays for kinase activities toward the CREB and NGFI-B protein substrates were conducted under similar conditions as described in the text using 1 μg of substrate protein per reaction. These reactions were terminated by the addition of Laemmli sample buffer and boiling for 5 min, and the proteins were resolved by SDS-PAGE. The 32P-labeled proteins were detected by autoradiography. Bisindolylmaleimide V (BIM) (LC Laboratories, Woburn, MA) was added to aliquots of Mono Q fractions containing Fos kinase to the final concentrations indicated in the text.

Western Blot Analysis—50-μl aliquots of Mono Q HR5/5 column fractions were resolved by SDS-PAGE and transferred onto positively charged polyvinylidene fluoride membrane (NEN Life Science Products, 0.9 A for 2 h in 15 mM Tris, pH 8.3, 100 mM glycine, and 9% methanol. The blots were blocked with 5% bovine serum albumin in Tris-buffered saline and probed with anti-pp90^ras^2 (Santa Cruz Biotechnology, Santa Cruz CA), anti-pp90^ras^2 (Upstate Biotechnology, Inc., Lake Placid NY), or anti-pp90^ras^2 (gift from Dr. David Mollar) antibodies at a 1:1000 or 1:500 dilution, respectively. The phospho-specific antibody was used at 1:20,000 (Promega, Madison, WI). Immunoreactivity was detected using enhanced chemiluminescence as recommended by the manufacturer (Pierce).

Production of Bisindolylmaleimide-Sepharose—CNBr-Sepharose (Pharmacia, Uppsala, Sweden) was washed with HCl and activated by brief incubation with carbonate, pH 8. BIM was added to a final concentration of 20 μM with activated CNBr-Sepharose and incubated for 15 min at 25 °C in the dark. The resin was then washed three times with approximately 100 volumes of 20 mM Tris, pH 7.4, and unreacted groups were quenched by incubation with 100 mM glycine for 30 min. The resin was then washed with approximately 100 volumes 20 mM Tris, pH 7.4, 0.1% Tween 20 in 20 mM Tris, pH 7.4, five times for 15 min each. The unreacted BIM was then blocked by incubation with ampholines (Pharmacia, Uppsala Sweden) and washed once as described above. Fos kinase-containing samples were bound to BIM-Sepharose after first preclearing the samples with agarose beads by incubation in 150 mM NaCl, 20 mM Tris, pH 7.4, 1% Triton X-100, 1 mM EGTA, and 0.1 M orthovanadate. The resin was then washed five times with this buffer and once with high pressure liquid chromatography grade water (Sigma). Bound proteins were eluted from the resin using SDS-PAGE sample buffer and resolved on 9% SDS-PAGE.

Protein in Gel Digestion—Protein bands were excised from the silver-stained gels. Mincing gel pieces were washed with 25 mM NH_4HCO_3 in 50% acetonitrile, dried by speedvac, rehydrated in 25 mM NH_4HCO_3 solution containing trypsin, and digested overnight at 37 °C. Peptides were extracted by washing with high pressure liquid chromatography grade water followed by three washes with 50% acetonitrile, 5% trifluoroacetic acid at room temperature. The combined supernatants were dried down and redissolved in 50% acetonitrile, 5% trifluoroacetic acid prior to unseparated digests analysis. The peptide extracts were further desalted through a C18 column (0.3 mm inner diameter × 1 mm) (LC packing, San Francisco, CA).

Mass Spectrometric Analysis of the Peptides—MALDI and PSD molecular masses of all tryptic peptides were determined by analyzing 1/10th of unseparated digest using a Voyager DE-STR Biospectrometry Workstation (Perspective Biosystems, Inc.) with a delayed extraction MALDI mass spectrometer and operated in the reflector mode. Peptides were co-crystallized with equal volumes of matrices consisting of saturated solutions of α-cyano-4-hydroxycinnamic acid in 50% acetonitrile, 1% trifluoroacetic acid. All MALDI spectra were internally calibrated by using trypsin autolysis products. Peptide masses were submitted for protein mass data base searching. The matched and unmatched masses were subjected to post-source decay (PSD) analysis on the same instrument to determine the peptide sequence and any possible sites of modification.

Liquid Chromatography-Mass Spectroscopy—The capillary high pressure liquid chromatography system (180 mm × 15 cm C18 column) was interfaced to a Mariner Biospectrometry workstation (Perspective Biosystems, Inc.) equipped with an electrospray source and orthogonal acceleration time-of-flight mass analyzer. The unseparated digests were directly injected onto C18 capillary column (180 mm × 15 cm). The tryptic peptide masses were also determined for peptide mapping.

Data Base Searching and PSD Data Interpretation—All the measured tryptic peptide masses measured either by MALDI-TOF or liquid chromatographic-electron spray were analyzed using MS-Fit program developed by K. Clauser and P. Baker.

RESULTS

Fos Kinase Activation Is Dependent on p21^ras^ Activity—To test the involvement of p21^ras^ in NGF-stimulated Fos kinase activation, we used PC12 cells expressing a dominant negative

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2 The MS-Fit and MS-Tag program can be found on web site http://prospector.ucsf.edu.
form of p21ras (GSRasDN6 cells) under the control of the dexamethasone-sensitive murine mammary tumor virus promoter (15). NGF treatment of these cells resulted in Fos kinase activation. However, following dexamethasone treatment, Fos kinase activation was refractory to NGF stimulation (Fig. 1). Control studies demonstrated that dexamethasone treatment of wild-type PC12 cells did not affect Fos kinase activation (data not shown). The observation that the presence of a dominant negative form of p21ras within PC12 cells blocked Fos kinase activation demonstrates that Fos kinase lies downstream of p21ras in the NGF-induced signaling cascade.

Fos Kinase Is a Downstream Target of the MAP Kinase Cascade—To test whether Fos kinase was a downstream effector of the Raf/MEK/ERK/MAP kinase pathway, we stimulated PC12 cells with NGF in the presence or absence of the MEK inhibitor, PD98059 (18). Exposure of cells to PD98059 abolished Fos kinase activation (Fig. 2), demonstrating that Fos kinase is a downstream element within the MAP kinase pathway. Fos kinase eluted in fraction 24 (180 mM NaCl). Fos kinase activation was resistant to both wortmannin and LY294002 treatment demonstrating that Fos kinase activation is not dependent on the activity of phosphoinositol 3-kinase (data not shown). Fos kinase activation is also resistant to inhibition by rapamycin further demonstrating that it is not involved in the pp70s6k pathway (3).

Characterization of Fos Kinase Using Pharmacological Agents—Fos kinase was initially identified as an NGF-stimulated protein kinase activity capable of phosphorylating serine 362 of c-Fos (3, 4). We screened a panel of commercially available protein kinase inhibitors for their ability to inhibit Fos kinase activity in vitro. We tested these agents at twice the IC50 values for their identified enzyme targets. Fos kinase activity was resistant to the PKC inhibitors chelerythrine Cl2 (19) and calphostin C (20), the CK II inhibitor 5,6-dicholor-1-b-D-ribofuranosylbenzimidazole (DRB), 12 μM; 1-chloro-3-tosylamido-7-amino-2-heptanone (TLCK), 50 μM; BIM, 32 mM; Gö6976, 20 μM; chelerythrine Cl, 1.2 μM (±S.D. of triplicate determinations of individual reactions). The data are expressed as Relative 32P incorporated into Fos peptide expressed as percent of activity measured in control reactions carried out in the absence of added inhibitors.

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Fig. 1. Fos kinase activation is dependent on p21ras activity. The PC12 cell line GSRasDN6 that expresses a dominant negative mutant of p21ras under the control of the murine mammary tumor virus promoter was incubated 24 h with dexamethasone (Dex) or left untreated. The cells were then either stimulated with 50 ng/ml NGF for 5 min or left unstimulated. Fos kinase was assayed following partial purification by Mono Q chromatography. Fos kinase activity is expressed as mean counts/min incorporated into the Fos peptide (±S.D. of triplicate determinations). The data presented are representative of three separate experiments.

Fig. 2. Fos kinase activation is inhibited in the presence of the MEK inhibitor PD98059. PC12 cells were serum-starved for 16 h, harvested in phosphate-buffered saline, and either left unstimulated, or stimulated with 50 ng/ml NGF for 5 min in the presence (open circles) or absence (solid squares) of 25 μM PD98059. Lysates were prepared from PC12 cells and fractionated by Mono Q chromatography and assayed for Fos kinase activity using the Fos peptide as a substrate. Fos kinase activity is expressed as mean counts/min incorporated (±S.D. of triplicate determinations). These data presented are representative of three separate experiments.
two inhibitors exhibiting IC50 values for Fos kinase between 15 and 20 nM (see Fig. 9B). Both BIM (24) and Go6976 (25) have been held to be potent and selective inhibitors of PKC exhibiting an IC50 for these enzymes of between 15 and 20 nM. However, detailed analysis has revealed that BIM is equally effective in inhibiting the ribosomal S6 kinases, pp90rsk2 and pp70s6k (26), probably due to the homology between these enzymes and PKC within their ATP-binding subdomains. The possibility that Fos kinase was a classical or novel PKC isoform was ruled out by its activity in the presence of EGTA and the other PKC inhibitors described above. The sensitivity of Fos kinase to BIM and the dependence on MEK activity suggested the possibility that it might be an S6 kinase family member. Taylor et al. (4) demonstrated that Fos kinase was distinct from pp90rsk1 and pp70rsk6. Comparison of the Mono Q elution profiles of Fos kinase activity with that of the identified members of this family, pp90rsk1, pp90rsk2, and pp90rsk3, revealed that Fos kinase activity eluted at 180 mM NaCl, whereas pp90rsk2 eluted at 70 mM NaCl, pp90rsk3 eluted at 100 mM (Fig. 4), and pp90rsk3 eluted at 240 mM NaCl. Taken together, these data suggested that Fos kinase represented a novel S6 kinase or PKC family member.

Isolation of Fos Kinase Using Bisindolylmaleimide-Sepharose—BIM potently inhibited Fos kinase which demonstrated its high affinity and selective binding to Fos kinase. This finding raised the possibility that BIM could be used as an affinity ligand to facilitate purification of the enzyme. BIM was conjugated to CNBr-Sepharose to create an affinity resin. This resin was shown to deplete Fos kinase from peak Mono Q fractions. A BIM-insensitive Fos peptide kinase eluting at 70 mM NaCl (fraction 18) was used as a negative control to test the specific nature of Fos kinase binding to the BIM-Sepharose. CNBr-Sepharose conjugated to tryptophan was used as a control to demonstrate specificity because the structural backbone of both BIM and tryptophan consisted of indole groups (Fig. 5). Fos kinase is a phosphoprotein (3), allowing its binding to the BIM-Sepharose to be monitored by metabolic labeling of the protein with [32P]orthophosphate. PC12 cells were labeled with [32P]orthophosphate, harvested, and split into two pools; one pool of cells was stimulated with NGF for 5 min and the other cells remained unstimulated. Lysates from these cells were fractionated by Mono Q chromatography, and the resulting fractions were assayed for Fos kinase activity using the Fos peptide as a substrate. Aliquots of these fractions were incubated with BIM-Sepharose in parallel as described under “Methods and Materials.” Phosphoproteins that remained bound to the resin after washing were resolved by SDS-PAGE. Autoradiography of the resulting gel revealed the presence of a 32P-labeled protein with a mass of 88 kDa that co-eluted from the Mono Q column with Fos kinase activity (Fig. 4). Furthermore, this 32P-labeled protein was detected in the NGF-stimulated cell lysates. In similar experiments using [35S]methionine-labeled protein, it was shown that this 88-kDa protein binds in both the stimulated and unstimulated samples demonstrating that it becomes phosphorylated upon NGF stimulation (Fig. 6B).

To verify that this 88-kDa phosphoprotein was Fos kinase, peak Mono Q fractions from 32P-metabolically labeled and NGF-stimulated PC12 cell lysates were size-fractionated by gel filtration using a Superose 12 column. The resulting gel filtration fractions were then assayed for Fos kinase activity in parallel with incubation of aliquots with BIM-Sepharose as described above. Comparison of the 32P-labeled proteins bound to the BIM-Sepharose and the Fos kinase activity in the corresponding gel filtration fractions again revealed the presence of an 88-kDa band that co-eluted with the Fos kinase activity from the Superose 12 column. These data demonstrated that BIM-Sepharose is a useful reagent for the rapid isolation of Fos kinase (Fig. 6C).
mass spectroscopy to determine their individual masses. The results of this analysis revealed that the isolated protein possessed 20 tryptic peptides in common with pp90\textsuperscript{rsk2} (Fig. 7 and Table 1).

These data were consistent with the earlier data demonstrating that Fos kinase was dependent on p21\textsuperscript{ras} and MEK for its activation. However, the result obtained from the mass spectrographic analysis of Fos kinase contradicted earlier results obtained from Western blot analysis that suggested Fos kinase and pp90\textsuperscript{rsk2} were distinct (see Fig. 4). We therefore probed immunoblots of Mono Q fractions from NGF-stimulated PC12 cell lysate with two different pp90\textsuperscript{rsk2} antibodies. The antibody used initially in this study was raised against an internal region of pp90\textsuperscript{rsk2} (residues 602–615) and is referred to as anti-pp90\textsuperscript{rsk2,602–615}. By using this antibody we found pp90\textsuperscript{rsk2} immunoreactivity (fractions 18–22) was clearly resolved from the fractions that contained peak Fos kinase activity (fractions 24–28) (Fig. 8A and Fig. 4). However, when a different antibody raised against the extreme carboxyl terminus of pp90\textsuperscript{rsk2} (residues 722–740), referred to as anti-pp90\textsuperscript{rsk2,722–740}, was used, two peaks of immunoreactivity were detected. One immunoreactive peak corresponded to the peak of activity detected by anti-pp90\textsuperscript{rsk2,602–615} eluting at a NaCl concentration of 90 mM (fractions 18–22) and the other to the peak of Fos kinase activity at a NaCl concentration of 180 mM (fractions 24–28). These data demonstrated that pp90\textsuperscript{rsk2} exists in NGF-stimulated cells as two immunologically and chromatographically distinct species.

Immunoprecipitation using anti-pp90\textsuperscript{rsk2,722–740} efficiently removed Fos kinase activity from Mono Q fractions 24–28, and the activity was recovered in the immunoprecipitate. Likewise, immunoprecipitation from the same fractions using the anti-pp90\textsuperscript{rsk2,602–615} antibody showed similar results (Fig. 8B), with very little Fos peptide activity detected in immune complexes from fractions 18–22 when using either antibody (data not shown). This suggests that the protein species detected by these antibodies within fractions 18–22 does not represent a catalytically active form of pp90\textsuperscript{rsk2}, whereas the species within fractions 24–28 is enzymatically active. A small amount of pp90\textsuperscript{rsk1} activity was observed in anti-pp90\textsuperscript{rsk2} immunoprecipitates from both peak I and peak II. This suggests that both peaks of Fos peptide activity are largely due to enzymes other than pp90\textsuperscript{rsk1}.

It had previously been asserted that the Fos peptide kinase activity that eluted at 70 mM NaCl arose from pp90\textsuperscript{rsk1} (4). However, this is unlikely since most of this activity is neither inhibited in the presence of BIM nor recognized by the pp90\textsuperscript{rsk2} antisera in immunoprecipitation reactions. Together, these data further demonstrate that pp90\textsuperscript{rsk2} is capable of using the Fos peptide as a substrate but that a majority of the NGF-stimulated Fos peptide activity observed in these column fractions arises from pp90\textsuperscript{rsk2}.

**Fos Kinase and pp90\textsuperscript{rsk2} Are Distinct from CREB Kinase**—There has been considerable confusion over the identity of protein kinases that phosphorylate the transcription factors c-Fos, CREB, and NGFI-B (8). We fractionated cellular lysates from NGF-stimulated PC12 cells by anion exchange chromatography and assayed for CREB kinase activity using a peptide substrate, CREBtide (13, 14) in parallel with assays using the Fos peptide as a substrate. CREBtide is a peptide encompassing the regulatory serine phosphorylation site, serine 133. The major peak of CREBtide kinase activity eluted at 100 mM NaCl and was clearly resolved from the peak of Fos peptide kinase activity that eluted at a NaCl concentration of 180 mM NaCl (Fig. 9A), suggesting that Fos kinase was distinct from the CREBtide phosphorylating activity. Most strikingly, neither of the pp90\textsuperscript{rsk2} antisera used in this study was capable of deplet-
FIG. 6

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A

B

C

NGF Stimulated

Unstimulated

NGF Stimulated

Unstimulated

FIG. 6
ing significant amounts of the CREBtide activity present within these fractions (data not shown). In contrast the Fos kinase activity was removed upon immunoprecipitation with the pp90\textsuperscript{rsk2} antibodies. These results demonstrate that different enzymes are responsible for phosphorylating the Fos peptide and CREBtide within fractions 24-26 and that the pp90\textsuperscript{rsk2}-immunoreactive species present in fractions 18–22 did not possess significant activity toward either the Fos peptide or the CREBtide. Consistent with this, the anti-pp90\textsuperscript{rsk2,602–615} immunoreactive protein also failed to co-elute with the peak of CREBtide kinase activity. Therefore, these data demonstrate that Fos kinase was distinct from the major CREBtide kinase. Significantly, when recombinant CREB protein was used as a substrate (Fig. 9B), the peak of activity phosphorylating this substrate was chromatographically distinct from that phosphorylating the CREBtide. These data indicate that the enzymes responsible for the phosphorylation of these substrates represent two independent enzyme activities.

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**Fos Kinase and NGFI-B Kinase I Represent the Same Enzyme Species—NGFI-B kinase I has been suggested to be pp90\textsuperscript{rsk2} based on its in vitro substrate specificity (8). Fos kinase and NGFI-B kinase I possess many features in common including their behavior on Mono Q and Superose 12 columns (4, 8). In addition, pp90\textsuperscript{rsk2} was capable of phosphorylating NGFI-B in immune kinase reactions.**

As an initial step in determining whether these two kinase activities arose from the same enzyme species, we assayed Mono Q column fractions of NGF-stimulated PC12 cell lysates with both Fos and NGFI-B substrates allowing a direct comparison of the behavior of these enzymes on this chromatographic resin. Fos kinase activity eluted from Mono Q columns at a NaCl concentration of 180 mM and constituted the major NGF-stimulated activity phosphorylating this substrate in the cell lysate. pp90\textsuperscript{rsk1} also phosphorylated the Fos peptide but was chromatographically distinct from Fos kinase activity.

**FIG. 6.** An 88-kDa protein co-fractionates with Fos kinase activity and binds to bisindolylmaleimide-Sepharose. PC12 cells were metabolically labeled with \textsuperscript{32}P and either stimulated with 50 ng/ml NGF for 5 min (solid circles) or left unstimulated (open circles). The cells were then lysed, and the lysates were fractionated by Mono Q chromatography. A, Fos kinase activity was assayed by phosphorylation of the Fos peptide and expressed as mean counts/min incorporated into Fos peptide (±S.D. of triplicate determinations of individual reactions) (upper panel). Aliquots of even-numbered fractions were incubated in the presence of BIM-Sepharose as described under “Materials and Methods” and washed. Proteins that stably associated with the BIM-Sepharose were resolved by SDS-PAGE and detected by silver staining (inset). Mass spectroscopic analysis of the trypsin-digested samples revealed 20 tryptic peptides identical to those predicted for pp90\textsuperscript{rsk2} (see Table I).

3 K. D. Swanson and G. E. Landreth, unpublished observations.

**FIG. 7.** Purification and mass spectroscopic analysis of Fos kinase. Fos kinase from 7 x 10\textsuperscript{6} NGF-stimulated PC12 cells was partially purified by anion exchange chromatography and then the peak fractions were pooled and incubated with BIM-Sepharose. The resin was washed extensively, and the associated proteins were resolved on SDS-PAGE and detected by silver staining (inset). Mass spectroscopic analysis of the tryptic peptides revealed 20 tryptic peptides identical to those predicted for pp90\textsuperscript{rsk2} (see Table I).
Identification of Fos kinase as pp90\textsuperscript{rsk2} using MALDI-TOF and -post-source decay analysis

Mass spectrometric analysis of tryptic-digested Fos kinase samples revealed 19 unique tryptic peptides identical to those predicted for pp90\textsuperscript{rsk2}.

| Peptide mass measured (Da) | 1/\text{ppm}^a | Location | Peptide sequence determined by PSD\textsuperscript{b} and/or consistent with mass of pp90\textsuperscript{rsk2} |
|---------------------------|---------------|----------|--------------------------------------------------|
| 856.5546                  | -8.6          | 668-675  | (R)LTAAVLR(H)                                    |
| 1047.5837                 | -10.8         | 49-57    | (K)IEIAITHVYK(E)                                 |
| 1132.5590                 | -8.4          | 659-667  | (K)MLHVDQHPQRL(L)                                |
| 1147.5789                 | -18.5         | 63-72    | (K)ADIWPQPELKR(V)                                |
| 1148.5511                 | 15.3          | 659-667  | (K)MLHVDQHPQRL\textsuperscript{1 Met-ox}       |
| 1165.6955                 | 143-151       | 515-525  | (K)LHYAPFERT(K)                                  |
| 1179.6339                 | -24.2         | 133-142  | (K)NGSPYFNPRG(S)                                  |
| 1193.5887                 | -5.7          | 713-723  | (K)GAMAYATSYALK(R)\textsuperscript{1 Met-ox}   |
| 1186.3644                 | -5.9          | 701-712  | (R)YQYHNPITLKD                                  |
| 1214.5377                 | -8.9          | 470-480  | (R)DPTEEEILR(Y)                                  |
| 1283.7143                 | -2.4          | 459-469  | (R)DGAGPDVEEIKR(H)                               |
| 1327.7075                 | -1.7          | 511-523  | (R)SNQPGTDievK(E)                               |
| 1340.7241                 | -5.0          | 414-425  | (K)LAAQAGDPVPQRTK(Y)                             |
| 1400.6869                 | -0.6          | 458-469  | (R)DGAGPDVEEIKR(D)                               |
| 1483.8056                 | -4.3          | 120-132  | (R)DRILEVNHPIVK(L)                               |
| 1522.8569                 | -4.2          | 368-383  | (R)KDSFGPAASSAHLPRF(G)                           |
| 1706.8351                 | -15.4         | 285-300  | (R)KLGMPQFLSAPQSLLM(G)                           |
| 1786.9163                 | -20.3         | 285-300  | (R)KLGMPQFLSAPQSLLM(R)                           |
| 1802.9347                 | -7.1          | 285-300  | (R)KLGMPQFLSAPQSLLM\textsuperscript{1 Met-ox} |
| 1823.9703                 | -8.6          | 193-208  | (R)DLKPSNYLVDWDESNGPESRI(D)                     |
| 2246.118                  | -5.2          | 539-558  | (R)DLKPSNYLVDWDESNGPESRI(D)                     |

Unknown peaks: 1538.8350, 1924.9293

\textsuperscript{a} Peptide mass differences between measured and theoretical values.
\textsuperscript{b} All underlined peptide sequences were determined by MALDI-DE-PSD.

from Fos kinase, eluting from the Mono Q column at a NaCl concentration of 70 mM as shown in Fig. 4. It should be noted that this activity peak consists largely of an uncharacterized Fos kinase species that is resistant to BIM inhibition (see Fig. 5) and therefore does not arise from the activity of pp90\textsuperscript{rsk2}. The major peak of NGFI-B kinase I activity, as assayed by the phosphorylation of the DNA binding domain-containing fragment of NGFI-B (9), co-eluted with the Fos kinase (pp90\textsuperscript{rsk2}) activity at a NaCl concentration of 180 mM NaCl (Fig. 10A). This demonstrated that Fos kinase and NGFI-B kinase I exhibit similar behavior on anion exchange chromatography. Moreover, both activities were equally sensitive to the presence of BIM in in vitro reactions (Fig. 10B).

Substrate competition assays were performed to determine if a single enzyme species was responsible for phosphorylating both the Fos peptide and NGFI-B substrates or if distinct enzymes were performing these functions (Fig. 10C). The Fos peptide competitively inhibited the phosphorylation of the NGFI-B fragment as a substrate in in vitro phosphorylation reactions. Together, these data support the conclusion that Fos kinase and NGFI-B kinase I are pp90\textsuperscript{rsk2}.

**DISCUSSION**

**Fos Kinase Is pp90\textsuperscript{rsk2}**—A major conclusion of this study is that the activity we have identified as Fos kinase is pp90\textsuperscript{rsk2}. The pp90\textsuperscript{rsk} family is comprised of three members, pp90\textsuperscript{rsk1}, pp90\textsuperscript{rsk2}, and pp90\textsuperscript{rsk3} (27). Members of this family are distinguishable by the presence of two non-identical kinase domains, an amino-terminal kinase domain that is related to protein kinase A and a carboxyl-terminal kinase domain that is most closely related to phosphorylase b kinase (28, 29). The amino-terminal domain is responsible for all known intermolecular phosphorylation events mediated by pp90\textsuperscript{rsk}. The carboxyl-terminal kinase domain is necessary for enzymatic activation of the amino-terminal kinase domain since mutation of a critical lysine residue within its ATP binding domain blocks the in vitro activation of the holo-enzyme (30, 31). pp90\textsuperscript{rsk1} is a major downstream target of the p42 and p44 MAP kinases, ERK1 and ERK2 (13, 32, 33). Several sites within pp90\textsuperscript{rsk1} have been identified that are phosphorylated in vitro by ERKs and in vivo upon stimulation with phorbol 12-myristate 13-acetate (34). It is not clear whether the other two members of the pp90\textsuperscript{rsk} family are activated in a completely analogous manner to pp90\textsuperscript{rsk2} since pp90\textsuperscript{rsk3} is not activated in vitro by ERK. Upon phosphorylation by ERK, pp90\textsuperscript{rsk2} then undergoes extensive autophosphorylation that is necessary for its full activation (34, 35). Once activated, pp90\textsuperscript{rsk2} is translocated into the nucleus (36) where its targets are thought to include NGFI-B (30), Fos (5), and CREB (14, 37). In this way, pp90\textsuperscript{rsk2} is thought to affect the action of growth factors through phosphorylation of these transcriptional modulators of gene expression.

**Fos Kinase Is NGFI-B Kinase I**—The present study identifies NGFI-B kinase I as pp90\textsuperscript{rsk2} and shows that it was indistinguishable from Fos kinase by several independent measures. The orphan retinoid superfamily receptor, NGFI-B, binds DNA in its unphosphorylated form, and phosphorylation of NGFI-B at serine 350 is sufficient for the abolition of its DNA-binding ability (9). It was initially suggested that NGFI-B kinase I and Fos kinase were distinct enzymes based on differences in substrate specificities (8). However, a direct comparison of these activities using the substrates used to initially characterize Fos kinase were distinct enzymes based on differences in substrate specificities (8). However, a direct comparison of these activities using the substrates used to initially characterize Fos kinase (8). However, a direct comparison of these activities using the substrates used to initially characterize Fos kinase.

**CREB Kinase Is Distinct from Fos Kinase**—We have shown that the enzymes that phosphorylate Fos peptide, CREBtide, and the CREB protein are distinct. This conclusion differs from that of Xing et al. (13) who interpreted the phosphorylation of the peptide substrate, CREBtide, as reflective of the activity of the same enzyme that phosphorylated the full-length CREB protein substrate. The present data demonstrate that these substrates are differentially phosphorylated by pp90\textsuperscript{rsk2} as well as by uncharacterized activities that preferentially phosphorylate the CREBtide substrate. Upon direct comparison of the elution profiles of these activities, we found that all three activities were resolved from each other upon Mono Q chromatography. These data demonstrate that CREBtide is preferentially phosphorylated by a kinase that is distinguishable from the CREB protein kinase. Significantly, the CREBtide peak we observed is not altered by NGF stimulation. The CREBtide
kinase was identified by Xing et al. (13) as being pp90
\textsuperscript{rsk2} following its chromatographic purification. The final step of purification involved resolving the protein on an SDS-PAGE gel into which CREBtide was co-polymerized in order to detect the purified protein kinase by an in gel kinase assay. This assay revealed the presence of three distinct kinase activities of 90, 55, and 45 kDa. It is therefore unclear which of these species represents CREB kinase. Furthermore, the partially purified CREBtide kinase from NGF-stimulated PC12 cells identified by Ginty et al. (14) using this in gel kinase assay was 105 kDa. The more purified CREBtide kinase isolated from phorbol 12-myristate 13-acetate-stimulated T546 cells was reported to be 90 kDa. The most harmonious explanation is that pp90\textsuperscript{rsk2} was present in peak CREBtide kinase-containing fractions (13).

We found that the peak of pp90\textsuperscript{rsk2} immunoreactive species identified by the pp90\textsuperscript{rsk2,602–615} antibody eluted from Mono Q columns near the CREBtide activity (80 mM NaCl) but prior to the elution of Fos kinase activity (180 mM NaCl). However, when a second antibody, pp90\textsuperscript{rsk2,722–740}, was applied to these immunoblots it identified two immunoreactive peaks within these Mono Q fractions. One of these peaks corresponded to the peak observed with pp90\textsuperscript{rsk2,602–615}, whereas the second peak of immunoreactivity closely corresponded to the peak of Fos kinase activity within these column fractions. The pp90\textsuperscript{rsk2,602–615} antibody was also capable of depleting Fos kinase activity within the peak fractions from the fractions. However, neither antibody was capable of depleting activity from the peak fractions containing CREBtide activity. Thus, Fos kinase, isolated by two different protocols, BIM resin affinity chromatography and pp90\textsuperscript{rsk2} immunoprecipitation, was shown to be pp90\textsuperscript{rsk2}. Therefore, these data show that Fos kinase represents pp90\textsuperscript{rsk2} and that the CREBtide phosphorylating activity identified by Ginty et al. (14) is another kinase.

![Graph of CPM Incorporated into Fos Peptide](image)

**FIG. 8.** Two pp90\textsuperscript{rsk2} antibodies react differently on Western blots of Mono Q fractionated PC12 cell lysates. PC12 cells were harvested after 16 h serum starvation and either stimulated with 50 ng/ml NGF for 5 min or left unstimulated. The cells were then lysed, and the lysates were fractionated by Mono Q chromatography. Aliquots of even-numbered fractions were assayed for the presence of Fos kinase activity (A) and subjected to Western blot analysis using the indicated antibody against pp90\textsuperscript{rsk2} (B). Aliquots of fractions containing the pp90\textsuperscript{rsk2,602–615} immunoreactive species (peak I) or Fos kinase activity (peak II) were subjected to immunoprecipitation with the indicated antisera (C). The supernatants and pellets were assayed for the presence of Fos kinase activity which is expressed as the mean counts/min incorporated (±S.D. of triplicate determinations).
is significant to note in support of this conclusion that the peak of CREB kinase activity reported by Greenberg and colleagues (13) was seen to co-elute with protein recognized by the pp90\(^{602–615}\). It remains enigmatic why the pp90\(^{602–615}\) antibody fails to recognize an epitope in Fos kinase present in Mono Q fractions on Western blots. One possibility is that the two peaks of immunoreactivity represent two distinct pp90\(^{722–740}\) isoforms that differ in their primary structure. However, mass spectrometric analysis of the protein isolated by BIM-Sepharose or immunoprecipitated from Fos kinase peak fractions using the pp90\(^{602–615}\) antibody revealed the presence of a peptide whose mass corresponds to the peptide used to raise the pp90\(^{602–615}\) antibody. Another possibility is that the antigen-antibody interaction is sensitive to the phosphorylation state of the epitope. Whether this site present within the carboxyl terminus is phosphorylated during growth factor stimulation of pp90\(^{62}\) activity is not known. However, this site is neither phosphorylated in vivo during pp90\(^{62}\) activation by phorbol 12-myristate 13-acetate nor by ERK in in vitro reactions (34), and treatment with alkaline phosphatase does not alter the ability of this antibody to detect this protein on Western blots (data not shown). This suggests the possibility of another modification. The presence of a potential proline-directed phosphorylation site within the sequence used to raise the pp90\(^{62}\) antibody suggests the possibility that a phospho-directed proline isomerase, such as PIN1 (38), may induce a conformational change in the enzyme subsequent to its phosphorylation by MAP kinase. In this way, even dephosphorylation may not allow the protein to be recognized on the blot. It is also possible that the activation induces phosphorylation of the protein that results in its renaturation on the blotting membrane in a manner that masks the anti-

![FIG. 9. Fos kinase and CREB kinase activities possess distinct elution profiles on Mono Q columns.](image)

**A**. Fos kinase and CREB kinase activities were assayed by phosphorylation of either the Fos peptide (squares) or CREB peptide (circle) substrates, respectively, and expressed as mean counts/min incorporated (± S.D. of triplicate determinations). B. CREB kinase within these fractions was detected by incorporation of \(^{32}\)P into CREB protein. Phosphorylated CREB was resolved on SDS-PAGE and detected by autoradiography.

**FIG. 10. Fos kinase activity co-elutes with NGFI-B kinase I upon Mono Q chromatography.** PC12 cells were serum-starved for 16 h and either stimulated with 50 ng/ml NGF (solid lines) or left unstimulated (dashed lines). A. Lysates were prepared from PC12 cells and fractionated by Mono Q chromatography. The resulting fractions were assayed using either Fos peptide (open circles) or NGFI-B (solid squares) substrates, respectively. Fos kinase activity is expressed as mean counts/min incorporated. B. The ability of the peak Fos kinase activity to phosphorylate either Fos peptide (open circles or solid line) or NGFI-B (solid squares and dashed line) in the presence of increasing concentrations of BIM was assayed by incorporation of \(^{32}\)P into the Fos peptide and expressed as mean counts/min (± S.D. of triplicate determinations of individual reactions). Phosphorylated NGFI-B was resolved on SDS-PAGE, detected by autoradiography, excised from the gel, and incorporated \(^{32}\)P determined by Cerenkov counting. The observed IC\(_{50}\) of BIM for both Fos kinase and NGFI-B kinase is approximately 20 nM. C. NGFI-B kinase I activity was assayed in the presence of the indicated concentrations of the Fos peptide using 20 \(\mu\)g/ml NGFI-B as a substrate. Phosphorylated NGFI-B was resolved on SDS-PAGE and detected by autoradiography.

pp90\(^{62}\)\(^{602–615}\) epitope. Such a conformational change may be important to NGF-induced pp90\(^{62}\) activity and may account for the apparent interference in antibody recognition on
Transcription Factor Phosphorylation by pp90rsk2

Little is known about the physiologic roles played by pp90rsk1 or pp90rsk3 isoforms. However, various mutations within the X-linked pp90rsk2 locus result in Coffin-Lowry syndrome in humans. Genetic defects that cause this syndrome include point mutations demonstrated to abolish kinase activity in the encoded protein as well as large deletions of the pp90rsk2 coding region (39). These findings demonstrate that loss of pp90rsk2 kinase activity and not the loss of a specific structural function provided by this protein is the important factor in the production of the Coffin-Lowry phenotype. This syndrome is marked by progressive skeletal dysmorphisms and progressive psychomotor retardation (40). This linkage of pp90rsk2 to Coffin-Lowry is significant because it strongly suggests that despite the high levels of conservation between the different pp90rsk family members, pp90rsk2 performs a unique function necessary for maintenance of more differentiated cell populations that it cannot be adequately substituted by the other related family members, pp90rsk1 or pp90rsk3. Alternatively, the pp90rsk2 activity may be localized to different compartments in the cell or possess different substrate specificities than the other two isoforms. The observation that pp90rsk3 is not activated by ERK raises the possibility that the pp90rsk isoforms may be controlled by different activating stimuli.

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