B-Raf-Dependent Regulation of the MEK-1/Mitogen-Activated Protein Kinase Pathway in PC12 Cells and Regulation by Cyclic AMP

RICHARD R. VAILLANCOURT, ANNE M. GARDNER, AND GARY L. JOHNSON*

Division of Basic Sciences, National Jewish Center for Immunology and Respiratory Medicine, Denver, Colorado 80206, and Department of Pharmacology, University of Colorado Medical School, Denver, Colorado 80262

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Growth factor receptor tyrosine kinase regulation of the sequential phosphorylation reactions leading to mitogen-activated protein (MAP) kinase activation in PC12 cells has been investigated. In response to epidermal growth factor, nerve growth factor, and platelet-derived growth factor, B-Raf and Raf-1 are activated, phosphorylate recombinant kinase-inactive MEK-1, and activate wild-type MEK-1. MEK-1 is the dual-specificity protein kinase that selectively phosphorylates MAP kinase on tyrosine and threonine, resulting in MAP kinase activation. B-Raf and Raf-1 are growth factor-regulated Raf family members which regulate MEK-1 and MAP kinase activity in PC12 cells. Protein kinase A activation in response to elevated cyclic AMP (cAMP) levels inhibited B-Raf and Raf-1 stimulation in response to growth factors. Ras · GTP loading in response to epidermal growth factor, nerve growth factor, or platelet-derived growth factor was unaffected by protein kinase A activation. Even though elevated cAMP levels inhibited Raf activation, the growth factor activation of MEK-1 and MAP kinase was unaffected in PC12 cells. The results demonstrate that tyrosine kinase receptor activation of MEK-1 and MAP kinase in PC12 cells is regulated by B-Raf and Raf-1, whose activation is inhibited by protein kinase A, and MEK activators, whose activation is independent of cAMP regulation.

Receptor tyrosine kinases for nerve growth factor (NGF), basic fibroblast growth factor (bFGF), and platelet-derived growth factor (PDGF) have been shown to induce a neuronal differentiation response in PC12 pheochromocytoma cells (14, 17, 38). In contrast, the epidermal growth factor (EGF) receptor tyrosine kinase expressed in PC12 cells induces a proliferative response but not differentiation (14, 20, 36). The failure of the EGF receptor to induce the PC12 cell differentiation program may result, in part, from a difference in the kinetics of regulation of specific signal transduction pathways (29, 34) and the ability to induce the phosphorylation on tyrosines of specific proteins relative to that observed with the NGF, bFGF, and PDGF receptors (35). What is clear is that an overlapping set of signal transduction pathways are activated by NGF, bFGF, PDGF, and EGF (5, 17, 25, 33, 43, 47). Inhibition of Ras signaling by expression of a dominant negative Ras protein Ha-Ras(Asn-17) inhibited the ability of growth factor tyrosine kinases to induce PC12 cell differentiation (46, 52).

Recent studies have defined Raf-1 as an effector for Ras · GTP (48, 49, 54). Raf-1 is a serine-threonine protein kinase that phosphorylates and activates MEK-1 (8, 23). MEK-1 is a dual-specificity protein kinase which phosphorylates mitogen-activated protein kinase (MAPK) on both a tyrosine and a threonine, resulting in MAPK activation (1, 7, 21, 27, 40). Expression of the dominant negative Ha-Ras(Asn-17) polypeptide inhibited the growth factor activation of MAPK in PC12 cells (46, 52), suggesting a correlation between

the Ras-dependent regulation of MAPK and the induction of PC12 cell neuronal differentiation.

Since Raf-1 is an effector for Ras · GTP (48, 49, 54), the prediction is that Raf-1 is important in neuronal differentiation and MAPK regulation in PC12 cells. A constitutively active mutant Raf-1, when expressed in PC12 cells, induced neuronal differentiation but did not measurably activate MAPK (51, 52). This finding suggested that other serine-threonine protein kinases were involved in the growth factor receptor tyrosine kinase regulation of MEK-1, leading to MAPK activation.

Growth factor stimulation of PC12 cells has been shown to activate B-Raf, as measured by B-Raf autophosphorylation and phosphorylation of exogenous substrates (32, 44). In this report, we demonstrate that growth factor-activated B-Raf and Raf-1 from PC12 cells phosphorylate recombinant MEK-1. B-Raf phosphorylates and activates MEK-1 to phosphorylate recombinant MAPK, demonstrating a growth factor-dependent regulation of B-Raf and its ability to activate the MAPK pathway. In addition, B-Raf activity is inhibited by cyclic AMP (cAMP), analogous to Raf-1 (3, 6, 13, 37, 41, 53), yet the MEK-1/MAPK pathway remains unaffected. These results demonstrate that kinases in addition to Raf-1 are involved in the regulation of the MAPK pathway in PC12 cells.

MATERIALS AND METHODS

Cell culture. The human PDGF β receptor was introduced into rat pheochromocytoma PC12 cells by using the retroviral expression vector pLXSN as previously described (17). Geneticin-resistant clones were propagated and expression of the PDGF receptor was analyzed by immunoblotting. The PC12 cell clone WT 7, which expresses the wild-type PDGF β receptor, was used in the experiments presented herein. Only the PDGF β receptor and not the PDGF α receptor was detected in immunoblots from PC12 cell lysates (data not

* Corresponding author. Mailing address: Division of Basic Sciences, Goodman Building, K1007, National Jewish Center for Immunology and Respiratory Medicine, 1400 Jackson St., Denver, CO 80206. Phone: (303) 398-1772. Fax: (303) 398-1225.

shown). PC12 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) with 5% plasma-derived horse serum and 5% plasma-derived newborn calf serum (Cocalico Biologicals, Reamstown, Pa.) supplemented with 100 μ g of streptomycin per ml and 100 U of penicillin per ml (Gibco BRL). The cells were grown on 10-cm dishes coated with poly-L-lysine and maintained at 37°C in 7.5% CO₂. Rat 1a fibroblasts were maintained in DMEM with 5% newborn bovine calf serum and 5% bovine calf serum supplemented with streptomycin and penicillin as described above.

Immunoprecipitation. To assay Raf activation, immune complexes were incubated with recombinant kinase-inactive MEK-1 as a substrate (7, 11, 23). Cells were deprived of serum with 0.1% bovine serum albumin (BSA)–20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES; pH 7.2) in DMEM supplemented with 100 μ g of streptomycin per ml and 100 U of penicillin per ml for 18 to 20 h prior to growth factor stimulation. All growth factor stimulations were performed at 37°C for 5 min, unless stated otherwise, using EGF and PDGF-BB at 30 ng/ml and NGF at 100 ng/ml. Following stimulation, cells were washed twice with ice-cold phosphate-buffered saline (PBS) and lysed by scraping in 0.5 ml of lysis buffer. To immunoprecipitate Raf-1, cells were lysed with buffer consisting of 10 mM Tris (pH 7.4), 1% Triton X-100, 5 mM EDTA, 50 mM NaCl, 50 mM NaF, 0.1% BSA, 20 μ g of aprotinin per ml, 1 mM phenylmethylsulfonyl fluoride, and 2 mM Na₃VO₄. The insoluble material was pelleted by centrifugation at 10,000 $\times$ g for 10 min and discarded. For Raf-1 immunoprecipitates, nonspecific binding sites were cleared by preincubating the lysate with 25 μ l of a suspension of formalin-fixed *Staphylococcus aureus* in lysis buffer for 30 min at 4°C. The lysates were cleared by centrifugation for 10 min, and the supernatant was incubated with a rabbit antibody (1/100 dilution) raised against the C terminus of Raf-1 (CTLTTSPRL PVF; Santa Cruz Biotechnology) for 90 min at 4°C. To immunoprecipitate B-Raf, cells were lysed with buffer consisting of 70 mM β -glycerophosphate (pH 7.2), 100 μ M Na₃VO₄, 2 mM MgCl₂, 1 mM EGTA, 0.5% Triton X-100, 5 μ g of leupeptin per ml, 20 μ g of aprotinin per ml, and 1 mM dithiothreitol. The insoluble material was pelleted as described above. The soluble cell lysate was incubated with a rabbit antibody (1/100 dilution) raised against the C terminus of B-Raf (ASPKTPIQAGGYGAFPVH; Santa Cruz Biotechnology) for 90 min at 4°C. The immune complexes for either Raf-1 or B-Raf were then incubated for 30 min at 4°C with 25 μ l of 10% formalin-fixed *S. aureus* (Sigma), washed in lysis buffer, and then spun through a 600- μ l cushion of lysis buffer–10% sucrose in polystyrene tubes (10 by 75 mm) for 20 min at 2,500 rpm. The immune complexes were washed twice with 1 ml ice-cold radioimmunoprecipitation assay buffer (10 mM NaPO₄ [pH 7.0], 150 mM NaCl, 2 mM EDTA, 1% sodium deoxycholate, 1% Nonidet P-40, 0.1% sodium dodecyl sulfate [SDS], 20 μ g of aprotinin per ml, 50 mM NaF, 200 μ M Na₃VO₄, 0.1% β -mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride), twice with 1 ml of ice-cold PAN [10 mM piperazine-*N,N'*-bis(2-ethanesulfonic acid) (PIPES, pH 7.0), 100 mM NaCl, 20 μ g of aprotinin per ml] containing 0.5% Nonidet P-40, and once with 1 ml of ice-cold PAN. For each wash, the immune complexes were pelleted by centrifugation at 2,500 rpm for 5 min and resuspended by gentle vortexing. The pellet was resuspended in 0.5 ml of PAN, transferred to a 0.5-ml Eppendorf tube, and pelleted by centrifugation for 1 min at 5,000 rpm. This final pellet was resuspended in 10 to 20 μ l of PAN. For an in vitro kinase assay, 5 to 10 μ l of the PAN suspension was incubated with catalytically inactive recombinant MEK-1 (50 to 100 ng) and 10 to 30 μ Ci of [γ -³²P]ATP

(2,000 to 5,000 dpm/pmol) in buffer (20 mM PIPES [pH 7.0], 10 mM MnCl₂, 20 μ g of aprotinin per ml) in a final volume of 20 to 50 μ l for 15 min at 30°C. Reactions were stopped by the addition of an appropriate volume of 2 $\times$ Laemmli sample buffer. The samples were boiled for 3 min and subjected to SDS-polyacrylamide gel electrophoresis (PAGE) (9) and autoradiography.

Quantitation of cAMP. cAMP levels were measured from serum-starved cells maintained in six-well poly-L-lysine-coated dishes. Stimulations were performed in Hanks buffer as follows: 3-min stimulation with 50 μ M forskolin, 5-min stimulation with growth factors at the concentrations indicated above, or 3-min stimulation with forskolin and then 5-min stimulation with growth factor. cAMP levels were quantitated by using an Amersham radioimmunoassay kit as instructed by the manufacturer.

MAPK assay. Anion-exchange chromatography of PC12 cell extracts was performed at 4°C with a Pharmacia LKB Biotechnology Inc. fast protein liquid chromatography (FPLC) system essentially as previously described (17). PC12 cells were serum starved and stimulated as stated above. After stimulation, the cells were washed twice with ice-cold PBS, and then 650 μ l of lysis buffer was added as described above for B-Raf immunoprecipitations. Soluble extracts from two 10-cm-diameter dishes (500 μ l, 0.5 to 1 mg of protein) were applied to a Mono Q HR5/5 FPLC column equilibrated in buffer A (70 mM β -glycerophosphate [pH 7.2], 100 μ M Na₃VO₄, 2 mM MgCl₂, 1 mM EGTA, 1 mM dithiothreitol). The column was washed with 2 ml of buffer A, and the bound proteins were eluted with a 28-ml linear gradient of 0 to 350 mM NaCl in buffer A. Aliquots (20 μ l) of 1-ml fractions were mixed with 20 μ l of 50 mM β -glycerophosphate (pH 7.2)–100 μ M Na₃VO₄–20 mM MgCl₂–200 mM [γ -³²P]ATP (2,000 to 5,000 dpm/pmol)–50 μ g of IP₂₀ (TTYADFIASGRTGRRNAIHD)–1 mM EGTA–400 mM peptide containing EGF receptor residues 662 to 681 (RRELVEPLTPSGEAPNQALLR). After 15 min at 30°C, 10 μ l of 25% trichloroacetic acid was added, and 45 μ l of the reaction mix was spotted onto 2-cm squares of P-81 phosphocellulose paper (Whatman). The papers were washed three times (5 min each) in 400 ml of 75 mM phosphoric acid and dehydrated once by washing with acetone. The phosphocellulose papers were allowed to dry, then placed into 7-ml vials with 4 ml of scintillation fluid, and quantitated by liquid scintillation counting.

MEK-1 activation assay. MEK-1 was fractionated by Mono S chromatography, and recombinant kinase-inactive MAPK was used as a substrate to measure MEK-1 activity. Phosphorylated kinase-inactive MAPK was resolved by SDS-PAGE and quantitated with a PhosphorImager. These procedures were performed exactly as previously described (11).

Recombinant MEK-1 activation assay. To measure activation of recombinant wild-type MEK-1 by B-Raf, B-Raf immune complexes from PC12 cells stimulated with various growth factors were incubated with partially purified recombinant wild-type MEK-1 (100 ng) for 5 min in kinase buffer (20 mM PIPES [pH 7.0], 10 mM MnCl₂, 10 to 20 μ Ci of [γ -³²P]ATP, 20 μ g of aprotinin per ml) at 30°C. Partially purified recombinant kinase-inactive MAPK (300 ng) was added as a MEK-1 substrate, and the incubation continued for 10 min. As a control, lysates from EGF-stimulated cells were immunoprecipitated with preimmune rabbit serum (1/100; Cappel, Durham, N.C.) and used in the kinase assays. The reactions were terminated by the addition of an equal volume of 2 $\times$ Laemmli sample buffer, analyzed by SDS-PAGE and autoradiography, and quantitated with a PhosphorImager.

Ras activation. Accumulation of p21^{c-ras}·GTP was mea-

sured essentially as previously described (25, 39). Briefly, cells which were 80% confluent (10-cm-diameter dish) were washed once with Tris-buffered saline and serum starved to induce quiescence with 10 ml of phosphate-free DMEM (Gibco BRL) supplemented with 1 mM sodium pyruvate, 100 U of penicillin per ml, 100 μ g of streptomycin per ml, and 0.1% BSA. After 12 to 18 h, 30 μ l of 32 PO $_4$ (10 mCi/ml of H $_2$ O; NEN) was added to the medium, and the cells were maintained in an incubator for 6 to 12 h. The cells were stimulated with EGF, NGF, or PDGF at ambient temperature. After 5 min, the cells were washed twice with ice-cold PBS and then lysed with buffer containing 25 mM Tris (pH 7.5), 150 mM NaCl, 16 mM MgCl $_2$, 1% (vol/vol) Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 0.5% (vol/vol) aprotinin (Sigma), and 0.1 μ g of leupeptin per ml. The lysis buffer was supplemented with Ras monoclonal antibody Y13-259 (gift from Anne Harwood, National Jewish Center) at a final concentration of 20 μ g/ml. The lysates were immediately removed from the dish, and the nuclei and cell debris were removed by centrifugation at 14,000 $\times$ g for 10 min. The supernatant was incubated at 4°C for 1 h. Meanwhile, 3 μ l of the supernatant was quantitated by liquid scintillation counting. An equal amount of radioactivity was added to 30 μ l of goat anti-rat immunoglobulin coupled to agarose (Sigma). The lysate and agarose beads were rotated for 1 h at 4°C. The immune complexes were collected by centrifugation at 1,000 rpm for 1 min, washed with 1 ml of buffer consisting of 25 mM Tris (pH 7.5), 150 mM NaCl, 16 mM MgCl $_2$, and 1% (vol/vol) Nonidet P-40, and then gently agitated. After five washes, the immune complexes were resuspended with 20 μ l of elution buffer consisting of 2 mM EDTA, 0.2% SDS, and 2 mM dithiothreitol and then heated at 100°C for 3 min. The supernatants were collected by centrifugation at 14,000 $\times$ g for 5 min. Nucleotides were analyzed by thin-layer chromatography by applying 7 μ l to a polyethyleneimine-cellulose plate coated with fluorescent indicator (J. T. Baker Chemical Co.). Radioactive GDP and GTP were resolved by chromatography using 0.75 M KH $_2$ PO $_4$ (pH 3.5), and nonradioactive GDP and GTP were used as standards.

Immunoblotting. Soluble cell lysates were prepared as described above, and proteins were resolved by SDS-PAGE (8% gel) (9). Proteins were transferred to a nitrocellulose membrane for 1 h at 1 A, using 10 mM CAPS (cyclohexylaminopropanesulfonic acid; pH 11) and 10% methanol in a Hoefer Transfor apparatus. Proteins were stained with Ponceau S, and nonspecific sites were saturated for 1 h with blocking solution consisting of 2% nonfat dry milk in 0.1% Tween 20 plus Tris-buffered saline (TTBS). The Raf-1 and B-Raf C-terminal antisera used for immunoprecipitations were used at a dilution of 1/100 in blocking solution for 1 h. The membrane was washed three times with TTBS for 5 min each, then incubated with [125 I]protein A (NEN) in blocking solution for 1 h, and washed as described above. The immunoreactive proteins were visualized by autoradiography.

Analytical procedures. Gels and thin-layer chromatography plates were imaged and quantitated with a PhosphorImager (Molecular Dynamics, Sunnyvale, Calif.). The data and quantitation presented herein are representative figures from experiments performed at least three times.

RESULTS

Expression of B-Raf and Raf-1 in PC12 cells. B-Raf is a serine-threonine kinase having significant sequence homology to Raf-1 (42), but its regulation and function are poorly defined. B-Raf has been demonstrated to be expressed predominantly in neural tissues and the testes (45) but has been

FIG. 1. Immunoblot of Raf-1 and B-Raf from PC12 cells and Rat 1a fibroblasts. Cultured PC12 and Rat 1a cells were washed twice with ice-cold PBS and lysed with buffer containing 0.5% Triton X-100, and the soluble fraction was prepared as described in Materials and Methods. The proteins (250 μ g) were separated by SDS-PAGE (8% gel) and transferred to a nitrocellulose membrane. The molecular weight of Raf-1 and B-Raf was determined relative to low-range molecular weight markers from Bio-Rad. The Raf-1 (lanes a and c) and B-Raf (lanes b and d) carboxyl-terminal antisera were used at a dilution of 1/100, and [125 I]protein A was used to visualize the proteins.

found in a variety of other cell types. B-Raf can be expressed as either a 68- or 95-kDa polypeptide (32, 42, 44). Immunoblotting of PC12 cell lysates demonstrated that both the 68- and 95-kDa forms of B-Raf are expressed in PC12 cells (Fig. 1, lane b). Rat 1a cells, in which growth factors stimulate Raf-1 but not B-Raf activity, expressed only the 68-kDa B-Raf polypeptide (Fig. 1, lane d). Raf-1 is expressed in both PC12 and Rat 1a cells (Fig. 1, lanes a and c).

Growth factor regulation of Raf-1. Raf-1 has also been shown to be activated in response to the stimulation of growth factor receptor tyrosine kinases (22, 28, 30, 31). Raf-1 has been shown to phosphorylate and activate MEK-1 (MAPK kinase) (8, 23). We therefore examined the ability of Raf-1 to be activated following the stimulation of EGF, NGF, or PDGF receptors in PC12 cells, as measured by its ability to phosphorylate MEK-1 (Fig. 2). EGF, NGF, and PDGF stimulated Raf-1 activity between two- and threefold. These growth factors have been shown to stimulate MAPK activity in PC12 cells (17). Similarly, EGF stimulation of Rat 1a fibroblasts resulted in a 10-fold increase in Raf-1 activity (37). Since both PC12 and Rat 1a cells express Raf-1 and the 95-kDa polypeptide of B-Raf is unique to cells of neuronal origin (45), our study focused on the regulation of B-Raf by growth factor stimulation of the MAPK pathway in PC12 cells.

B-Raf is activated by growth factor stimulation of PC12 cells. To determine if B-Raf kinase activity was regulated by growth factor stimulation, PC12 cells were treated with EGF, NGF, or PDGF for 5 min, and B-Raf was immunoprecipitated from cell lysates. Addition of purified, recombinant kinase-inactive MEK-1 to the B-Raf immunoprecipitates demonstrated that MEK-1 was a substrate for the B-Raf kinase (Fig. 2A). Quantitation of MEK-1 phosphorylation in the *in vitro* kinase assay of B-Raf immunoprecipitates indicated that relative to controls, EGF, NGF, and PDGF stimulation of PC12 cells produced 5-, 3.5-, and 2-fold increases in MEK-1 phosphorylation, respectively. Immunoprecipitates obtained with preimmune serum had no MEK-1 phosphorylation activity (not shown).

Stimulation of protein kinase A has been shown to inhibit Raf-1 activation in response to growth factors (3, 6, 13, 37, 41, 53). In PC12 cells, growth factor-activated Raf-1 was inhibited by elevated levels of cAMP (Fig. 2). To determine if B-Raf activation was similarly regulated in PC12 cells, forskolin was

FIG. 2. Elevated levels of cAMP inhibit phosphorylation of kinase-inactive MEK-1 by growth factor-stimulated B-Raf and Raf-1 immunoprecipitated from PC12 cells. B-Raf and Raf-1 were immunoprecipitated from growth factor-stimulated-PC12 cells, and kinase-inactive MEK-1 was used as a substrate to quantitate kinase activity. PC12 cells were treated without (A and C) or with (B and D) 50 μ M forskolin for 3 min at 37°C and then without growth factor (lanes a, e, i, and m) and with NGF (lanes b, f, l, and p), PDGF (lanes c, g, k, and o), and EGF (lanes d, h, j, and n) for 5 min at 37°C. B-Raf (A and B) and Raf-1 (C and D) immune complexes were incubated with kinase buffer and [γ - 32 P]ATP for 15 min at 30°C. The reactions were terminated with 2 $\times$ Laemmli sample buffer, and the mixtures were heated for 5 min at 100°C. The proteins were separated by SDS-PAGE (8% gel) and visualized by autoradiography. Phosphorylated kinase-inactive MEK-1 was quantitated by PhosphorImager analysis, and the data are expressed relative to basal phosphorylation levels. The relative phosphorylation intensity of kinase-inactive MEK-1 is indicated below the autoradiograms. Sizes are indicated in kilodaltons.

used to stimulate cAMP synthesis and activate protein kinase A. Forskolin-treated PC12 cells were challenged with EGF, NGF, or PDGF, and B-Raf activity was assayed from immunoprecipitates. Forskolin treatment of PC12 cells for 3 min prior to growth factor stimulation strongly inhibited the activation of B-Raf, as measured by its ability to phosphorylate kinase-inactive MEK-1 (Fig. 2). Treatment of PC12 cells with forskolin elevated cAMP levels to 158 ± 8.2 pmol/mg of protein, compared with basal cAMP levels of 14 ± 1.5 pmol/mg of protein. cAMP levels were 13 ± 2.6 pmol/mg of protein after stimulation with growth factors, whereas forskolin pretreatment elevated cAMP levels to 170 ± 9.1 pmol/mg of protein. These data are consistent with previous data demonstrating that growth factors did not activate protein kinase A activity in PC12 cells (16).

Immunoprecipitates from quiescent and growth factor-stimulated cells which were labeled with [35 S]methionine showed that only the 95-kDa B-Raf form was immunoprecipitated by the B-Raf antiserum (not shown) even though both the 68- and 95-kDa B-Raf forms were immunoblotted from cell lysates. Also, antiserum against B-Raf specifically immunoblotted the 95-kDa protein isolated from B-Raf immunoprecipitates. The 68-kDa form of B-Raf was present at very low levels if at all in the immunoprecipitate. In addition, immunoprecipitates from quiescent and growth factor-stimulated cells had equal amounts of the 95-kDa B-Raf form, which demonstrates that EGF, NGF, and PDGF enhanced the B-Raf activity rather than increasing antibody accessibility to B-Raf.

Addition of [γ - 32 P]ATP to the immunoprecipitate resulted in the phosphorylation of the 95-kDa B-Raf polypeptide (Fig. 3A). Phosphorylation of the 95-kDa B-Raf polypeptide was increased six-, four-, and twofold relative to controls in immunoprecipitates of lysates from EGF-, NGF-, and PDGF-stimulated PC12 cells, respectively (Fig. 3C). This kinase activity most likely represents autophosphorylation of B-Raf (32, 44) and was not observed with immunoprecipitates obtained by using preimmune rabbit serum (Fig. 3B). The amounts of 95-kDa B-Raf were similar in immunoprecipitates from con-

FIG. 3. Autophosphorylation of growth factor-stimulated B-Raf immunoprecipitated from PC12 cells. PC12 cells were treated without growth factor (lanes a and e) or with EGF (lanes b and f), NGF (lanes c and g), or PDGF (lanes d and h) for 5 min at 37°C. Kinase reactions were performed in the absence of kinase-inactive MEK-1 as described for Fig. 2. (A) Autoradiogram of in vitro kinase reactions performed with B-Raf immune complexes. Sizes are indicated in kilodaltons. (B) Autoradiogram of in vitro kinase reactions performed with immune complexes derived from preimmune rabbit serum. (C) PhosphorImager quantitation of autophosphorylated B-Raf shown in panel A.

FIG. 4. Elevated levels of cAMP inhibit autophosphorylation of growth factor-stimulated B-Raf immunoprecipitated from PC12 cells. (A) PhosphorImager quantitation of the 95-kDa B-Raf shown in panels B and C. (B and C) Autoradiograms of nitrocellulose membrane in which PC12 cells were treated with or without 50 μ M forskolin for 3 min at 37°C and then treated without growth factor (lanes a and e) or with EGF (lanes b and f), NGF (lanes c and g), and PDGF (lanes d and h) for 5 min at 37°C. The autophosphorylation reaction was performed as described for Fig. 3. The samples were separated by SDS-PAGE (8% gel) and transferred to a nitrocellulose membrane. The position of the autophosphorylated protein migrated at the same position as the 95-kDa B-Raf which was identified by immunoblotting an adjacent lane containing a PC12 cell lysate as described for Fig. 1. Sizes are indicated in kilodaltons.

trol and stimulated cells, indicating that the phosphorylation resulted from activation of a kinase activity and not accessibility of B-Raf to antibody in cell lysates. Cumulatively, these findings demonstrate that B-Raf is activated in response to growth factor stimulation of PC12 cells (32, 44) and that B-Raf autophosphorylation and MEK-1 phosphorylation activities are similar in magnitude.

The 68- and 95-kDa B-Raf polypeptides from cell lysates were recognized by the B-Raf antiserum, as detected by immunoblotting. Interestingly, phosphorylation of the 68-kDa polypeptide was not detected in immunoprecipitates from either PC12 cells (Fig. 3 and 4) or Rat 1a fibroblasts (not shown). Even though autophosphorylation of the 68-kDa B-Raf polypeptide was not detected from growth factor-stimulated B-Raf immunoprecipitates, it is possible that the 68-kDa B-Raf polypeptide is activated in response to growth factors but that the activated protein is not recognized by the antiserum.

B-Raf phosphorylation of MEK-1 activates MEK-1. Recombinant wild-type MEK-1 was used in the B-Raf in vitro kinase assay to test whether its activity was changed in response to phosphorylation (Fig. 5). PC12 cells were challenged with growth factors for 5 min, and cell lysates were immunoprecipitated with B-Raf or control preimmune rabbit serum. Recombinant wild-type MEK-1 was incubated with the immune

FIG. 5. B-Raf phosphorylation leads to activation of wild-type MEK-1, which phosphorylates kinase-inactive MAPK. A portion of the B-Raf immunoprecipitate which was used in experiments described in the legend to Fig. 3 was used to phosphorylate recombinant wild-type (WT) MEK-1, which in turn activated and phosphorylated kinase-inactive MAPK. (A) Autoradiogram of a B-Raf immunoprecipitate which was incubated with 100 ng of recombinant wild type MEK-1 for 5 min with [γ - 32 P]ATP in kinase buffer at 30°C. Recombinant kinase-inactive MAPK (300 ng) was added, and the reaction was allowed to proceed for an additional 10 min and then terminated with 2 $\times$ Laemmli sample buffer. The samples were separated by SDS-PAGE (8% gel) with low-range prestained molecular weight markers (Bio-Rad). To optimize the separation between the recombinant MEK-1 and MAPK proteins, the samples were resolved until the 32-kDa marker reached the bottom of the gel. Size is indicated in kilodaltons. (B) Autoradiogram of growth factor-stimulated PC12 cells immunoprecipitated with preimmune rabbit serum (lanes e to h). Immune complexes were treated exactly as described for panel A. In lane i, wild-type MEK-1 and kinase-inactive MAPK were incubated with [γ - 32 P]ATP in kinase buffer in the absence of any immune complexes. Phosphorylation of wild-type MEK-1 was due to autophosphorylation which activated MEK-1 and resulted in a low level of phosphorylation of kinase-inactive MAPK. (C) PhosphorImager quantitation of wild-type MEK-1 and kinase-inactive MAPK phosphorylation shown in panel A.

binant wild-type MEK-1 was incubated with the immune complexes in kinase reaction buffer and [γ - 32 P]ATP. After 5 min, recombinant kinase-inactive MAPK was added, and the reaction was allowed to proceed for an additional 10 min. The B-Raf immunoprecipitates significantly increased the phosphorylation of MEK-1 relative to the preimmune serum. Quantitation of the results demonstrated a fourfold increase in MEK-1 phosphorylation in response to EGF and a concomitant increase in MAPK phosphorylation (Fig. 5C). NGF and PDGF stimulation of PC12 cells resulted in similar increases in B-Raf-mediated MEK-1 phosphorylation, also leading to increased MEK-1 activity (Fig. 5C, columns c and d). The omission of MEK-1 from the reaction prevented the increased MAPK phosphorylation (not shown), indicating that MEK-1 was required for the MAPK phosphorylation in B-Raf immunoprecipitates. When the kinase reaction was performed in the

FIG. 6. Elevated levels of cAMP inhibit phosphorylation and activation of wild-type MEK-1 and subsequent phosphorylation of kinase-inactive MAPK by B-Raf immunoprecipitates. PC12 cells were treated with or without 50 μ M forskolin for 3 min at 37°C and then treated without growth factor (lanes a and e) or with EGF (lanes b and f), NGF (lanes c and g), and PDGF (lanes d and h) for 5 min at 37°C. Lane i represents a coupled assay of a preimmune rabbit serum immunoprecipitate of EGF-treated PC12 cells in the absence of forskolin as shown in lane b. (A) Autoradiogram of a coupled assay in which B-Raf was immunoprecipitated from PC12 cells not pretreated with forskolin and incubated with wild-type (WT) MEK-1 and kinase-inactive MAPK as described for Fig. 5. Size is indicated in kilodaltons. (B) PhosphorImager quantitation of phosphorylated wild-type MEK-1 and kinase-inactive MAPK shown in panel A. (C) Autoradiogram of a coupled assay in which B-Raf was immunoprecipitated from PC12 cells pretreated with forskolin and incubated with wild-type MEK-1 and kinase-inactive MAPK. Size is indicated in kilodaltons. (D) PhosphorImager quantitation of phosphorylated wild-type MEK-1 and kinase-inactive MAPK shown in panel C.

absence of immune complexes (Fig. 5B, lane i), recombinant wild-type MEK-1 had a low level of autoactivation (11). However, this activity was similar to that observed with control preimmune rabbit serum (Fig. 5B, lanes e to h), demonstrating that B-Raf phosphorylation of MEK-1 is responsible for the increased MEK-1 activity (Fig. 5A, lanes a to d). The forskolin stimulation of cAMP synthesis and activation of protein kinase A also inhibited the activation of recombinant MEK-1 kinase activity by B-Raf immunoprecipitates from growth factor-stimulated PC12 cells (Fig. 6). In addition, the autophosphorylation of B-Raf in *in vitro* kinase assays was strongly inhibited by forskolin treatment prior to growth factor stimulation (Fig. 4). These results clearly demonstrate that protein kinase A activation inhibits growth factor stimulation of B-Raf activity. The findings also confirm that B-Raf kinase activity is required for the phosphorylation and activation of recombinant MEK-1.

Ras, MEK-1, and MAPK activation is not inhibited by cAMP. The inhibition of Raf-1 activation by forskolin-stimulated cAMP synthesis did not inhibit other signal functions of growth factor receptor tyrosine kinases (37). Measurement of GTP loading onto Ras in response to growth factor stimulation was unaffected in forskolin-treated PC12 cells (Fig. 7). This finding is similar to the inhibition of Raf-1 activation in fibroblasts. cAMP regulates the growth factor-stimulated MAPK activation pathway downstream of Ras and at the level, or upstream, of Raf-1 and B-Raf. This finding indicates that other MEK kinases must be involved in the regulation of MEK (MAPK kinase) in PC12 cells.

The stimulation of protein kinase A activity potentiates but is not required for NGF-induced differentiation (12, 15). PC12 cells treated with forskolin have a more robust differentiation than cells treated with NGF alone. Forskolin treatment of

PC12 cells does not inhibit EGF, NGF, or PDGF stimulation of MEK-1 (Fig. 8) or MAP kinase activity (Fig. 9). These findings sharply contrast with the dramatic inhibition of B-Raf activity by forskolin-stimulated cAMP synthesis. In contrast to recent findings, we do not see a significant activation of MEK-1 or MAPK activity in response to forskolin in the absence of growth factors (10). However, cAMP potentiates the growth factor-stimulated MEK-1 (Fig. 8) and p42 MAPK activities (Fig. 9). Thus, the enhanced activity of MEK-1 and p42 MAPK may be necessary for the potentiation of neurite outgrowth as mediated by cAMP. In other cell types, particularly fibroblasts, the cAMP-mediated inhibition of Raf-1 activity is correlated with an inhibition of MAPK activity (3, 6, 13, 37, 41, 53). Thus, there is a discordance between cAMP inhibition of B-Raf in PC12 cells, in which the MEK-1 and MAPK activities stimulated by growth factor tyrosine kinases remain unaffected. The results suggest that there may be additional growth factor-regulated MEK kinases in PC12 cells whose activation is not regulated by protein kinase A.

DISCUSSION

B-Raf is a serine-threonine protein kinase which is expressed predominantly in neuronal tissues and the testes (45). In contrast, Raf-1 is ubiquitously expressed in all cell types so far examined (18). B-Raf is a serine-threonine protein kinase related to Raf-1 (42). The 95-kDa form of B-Raf is activated in PC12 cells in response to growth factors that activate specific receptor tyrosine kinases. Reproducibly, EGF stimulated B-Raf activity to a greater extent than did NGF, which was similar in effect to PDGF. This is not simply a consequence of growth factor receptor number, because the PC12 cell clone

FIG. 7. Elevated levels of cAMP do not inhibit the accumulation of p21^{ras}·GTP in PC12 cells. PC12 cells were deprived of serum overnight and labeled with ³²PO₄ for 6 h in phosphate-free medium. The cells were pretreated with 50 μ M forskolin for 3 min and were treated without growth factor (lanes a and e) and with EGF (lanes b and f), NGF (lanes c and g), and PDGF (lanes d and h) for 5 min at ambient temperature. p21^{ras} was immunoprecipitated with rat monoclonal antibody Y13-259, and the bound guanine nucleotides were separated and quantitated. (A) PhosphorImager quantitation of radioactive GDP and GTP eluted from immunoprecipitated p21^{ras}. The data are presented as percent GTP relative to total GDP and GTP. (B and C) Radioactive GDP and GTP were separated by thin-layer chromatography and migrated at the same position as nonradioactive GDP and GTP, indicated by arrows.

that we used has approximately 10⁵ EGF and PDGF β receptors and only a few thousand NGF receptors (19, 20).

Previous reports have demonstrated that Raf-1 (52) and B-Raf (32, 44) become hyperphosphorylated, as measured by gel mobility shift and immunoblotting. However, the mobility shift of Raf-1 has been found not to correlate directly with activation of kinase activity and is not a good measure of Raf kinase phosphorylation of protein substrates (26, 51, 52). Our findings demonstrate that growth factor stimulation of PC12 cells increased B-Raf kinase activity to phosphorylate and functionally activate MEK-1.

B-Raf and Raf-1 have three conserved regions (CR) within their primary amino acid sequences (42). B-Raf and Raf-1 share 65% homology in CR1, which includes a putative zinc finger region. CR2 is a serine-threonine-rich domain sharing 47% homology between B-Raf and Raf-1. The COOH-terminal moiety is CR3 and encodes the kinase domain, which has 79% homology between B-Raf and Raf-1. The NH₂-terminal region of Raf-1 also interacts with Ras·GTP (48, 49, 54); we do not yet know if B-Raf interacts with Ras·GTP.

The ability of B-Raf and Raf-1 to be activated by growth

FIG. 8. Elevated levels of cAMP do not inhibit MEK-1 in growth factor-stimulated PC12 cells fractionated by cation-exchange chromatography. PC12 cells were treated with forskolin and growth factors as described for Fig. 6. Cell lysates were prepared and fractionated on a Mono S column, collecting 1-ml fractions. A 30- μ l aliquot from fractions 1 to 16 was assayed with [γ -³²P]ATP and kinase-inactive MAPK as a substrate. Phosphorylated proteins were separated by SDS-PAGE (10% gel) and quantitated by PhosphorImager analysis. Recombinant wild-type MAPK was autophosphorylated and used as a marker to identify the position of kinase-inactive MAPK on SDS-PAGE. The peak of MEK-1 activity, fractions 8 to 12, corresponded with MEK-1 immunoreactivity, whereas the peak consisting of fractions 3 to 5 most likely represents a MEK activity which is not immunoreactive with our C-terminal MEK-1 antiserum (not shown). This result is consistent with previous findings (11).

factor stimulation of PC12 cells and to be antagonized by protein kinase A suggests that regulatory factors in addition to Ras·GTP are involved in the control of Raf kinases. Evidence for the regulation of Raf kinases in addition to interaction with Ras·GTP has recently been found. For example, a requirement for phosphatidylcholine-specific phospholipase C (PC-PLC) activity for activation of Raf-1 has been documented (4). Presumably, the PC-PLC activity was regulating a kinase function involved in Raf-1 activation. It is unclear if B-Raf activation also involves a PC-PLC-like activity regulated by growth factor tyrosine kinases.

Activation of both B-Raf and Raf-1 in response to growth factors is inhibited by cAMP elevations and increased protein kinase A activity. Raf-1 appears to be phosphorylated at Ser-43 (53), but a similar consensus protein kinase A site is not present near the NH₂ terminus of B-Raf (42). Although there are other potential protein kinase A sites in the NH₂-terminal moiety of B-Raf, none are near the extreme NH₂ terminus. Therefore, protein kinase A activation similarly inhibits Raf-1 and B-Raf activation, but it is unclear whether this results from a direct phosphorylation of the two Raf kinases.

GTP loading onto Ras was robust with EGF, NGF, and PDGF. Ras GTP loading has been shown to be differentially regulated by neurotrophic and mitogenic factors. In PC12 cells stimulated with neurotrophic factors, p21^{ras} (29, 34) and MAPK (17) were persistently activated. Our findings in PC12 cells show that B-Raf and Raf-1 are coupling to tyrosine kinase response pathways. We have found that both Raf-1 and the 95-kDa form of B-Raf are expressed in human neutrophils and that both are activated in response to chemoattractants (2). Interestingly, B-Raf activation is prolonged relative to Raf-1 in neutrophils, indicating that the two Raf kinases are not identical in regulation.

A striking result is that protein kinase A activation has no

FIG. 9. Elevated levels of cAMP do not inhibit MAPK in growth factor-stimulated PC12 cells fractionated by anion-exchange chromatography. PC12 cells were treated with forskolin and growth factors as described for Fig. 6. Cell lysates were prepared and fractionated on a Mono Q column, collecting 1-ml fractions. A 20- μ l aliquot from fractions 10 to 22 was assayed with [γ - 32 P]ATP and the synthetic MAPK peptide substrate derived from the EGF receptor. Tyrosine-phosphorylated pp42 and pp44 MAPK were identified by immunoblotting with antiphosphotyrosine antiserum as previously described (17) and are indicated on the graph.

effect on the growth factor stimulation of MEK-1 and MAPK activity. This contrasts with the nearly complete inhibition of B-Raf activation following forskolin treatment of PC12 cells. The simplest explanation is that an additional MEK kinase (MAPK kinase kinase) activity is growth factor stimulated in PC12 cells and is refractory to protein kinase A inhibition. We have now found growth factor-stimulated Raf-independent MEK kinase activity in PC12 cells but have not identified the kinase at a molecular level (23a). Raf-independent MEK kinases have recently been identified in mammalian cells (24). A second explanation is that a B-Raf or Raf-1 activation occurs even with protein kinase A stimulation but that the activated protein is not stable to immunoprecipitation and *in vitro* kinase assay. This latter mechanism seems unlikely, but discrimination between these two possibilities will require both genetic and biochemical dissection of the MAPK pathway in PC12 cells. Additional regulatory inputs involving as yet unidentified modifiers of the MAPK pathway may also be required. What is clear is that the B-Raf/MEK-1/MAPK activation pathway in PC12 cells is regulated by protein kinase A in a similar fashion to Raf-1 in fibroblast systems in which Raf-1 is dominant. Inhibition of Ras activation completely inhibited MAPK activation in PC12 cells responding to growth factors (46, 52). Thus, activation of Raf-1, B-Raf, and any other MEK activators involved in the MAPK pathway in PC12 cells requires functional Ras signaling. Consistent with this hypothesis is the finding of Williams et al. (50) that MAPK activation occurs by Raf-1-dependent and -independent pathways.

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