Molecular Biology of the Cell
Vol. 3, 545–553, May 1992

The β-PDGF Receptor Induces Neuronal Differentiation of PC12 Cells

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Submitted January 21, 1992; Accepted March 26, 1992

Expression of the mouse β-PDGF receptor by gene transfer confers PDGF-dependent and reversible neuronal differentiation of PC12 pheochromocytoma cells similar to that observed in response to NGF and basic FGF. A common property of the PDGF, NGF, and basic FGF-induced differentiation response is the requirement for constant exposure of cells to the growth factor. To test the hypothesis that a persistent level of growth factor receptor signaling is required for the maintenance of the neuronal phenotype, we examined the regulation of the serine/threonine-specific MAP kinases after either short- (10 min) or long-term (24 h) stimulation with growth factors. Mono Q FPLC resolved two peaks of growth factor-stimulated MAP kinase activity that coeluted with tyrosine phosphorylated 41- and 43-kDa polypeptides. MAP kinase activity was markedly stimulated (~30-fold) within 5 min of exposure to several growth factors (PDGF, NGF, basic FGF, EGF, and IGF-I), but was persistently maintained at 10-fold above basal activity after 24 h only by the growth factors that also induce PC12 cell differentiation (PDGF, NGF, and basic FGF). Thus the β-PDGF receptor is in a subset of tyrosine kinase-encoded growth factor receptors that are capable of maintaining continuous signals required for differentiation of PC12 cells. These signals include the constitutive activation of cytoplasmic serine/threonine protein kinases.

INTRODUCTION

A significant hallmark of the PC12 cell differentiation program is the requirement for constant NGF or basic FGF exposure as indicated by the reversibility of the neuronal phenotype on removal of the growth factor from the medium. The implication is that persistent signals elicited by the growth factors are required to maintain the differentiated phenotype. An immediate signal produced on activation of NGF and basic FGF receptors that mediate neuronal differentiation of PC12 pheochromocytoma cells is increased tyrosine-phosphorylation of specific cellular proteins (Maher, 1988; Miyasaka *et al.*, 1991; Schanen-King *et al.*, 1991) and is mediated in part by the tyrosine kinase activity intrinsic to the respective receptor molecules (Lee *et al.*, 1989; Pasquale and Singer, 1989; Hempstead *et al.*, 1991; Kaplan *et al.*, 1991; Klein *et al.*, 1991). The v-src tyrosine kinase mimics the ability of the NGF and basic FGF receptors to induce neuronal differentiation (Alema *et al.*, 1985) and the intrinsic kinase activity of these gene products is critical for all aspects of their signal transduction (Ullrich and Schlessinger, 1990). Thus sustained signaling through these tyrosine kinases is an obvious mechanism by which NGF and basic FGF could maintain the differentiation response. However, enhanced protein tyrosine kinase activity, per se, is not sufficient for directing neuronal differentiation of PC12 cells because stimulation of the receptors for EGF or IGF-I, which are receptor protein tyrosine kinases expressed on PC12 cells (Huff *et al.*, 1981; Dahmer *et al.*, 1989), fail to signal neuronal differentiation and instead induce a modest mitogenic response (Huff *et al.*, 1981; Dahmer and Perlman, 1988).

The features of the NGF and basic FGF receptors relative to the EGF and IGF-I receptors that are involved in signal transduction leading to neuronal differentiation remain unclear. Elucidation of the key receptor tyrosine kinase signaling properties related to neuronal differentiation or cell growth of PC12 cells would be facilitated by a ligand-regulated receptor tyrosine kinase that is absent in parental PC12 cells and whose expression

by gene transfer directs neuronal differentiation. In this report, we demonstrate that the β-PDGF receptor directs reversible differentiation of PC12 cells similar to NGF and basic FGF receptors after expression by retroviral-mediated gene transfer.

Although immediate signaling through the growth factor receptor tyrosine kinases is dependent on their tyrosine kinase activity (Ullrich and Schlessinger, 1990), most of the phosphorylation events stimulated in response to growth factors occur on serine and threonine residues, indicating that selective regulation of protein serine/threonine kinase cascades by the various receptor protein tyrosine kinases offers a mechanism by which specificity for cellular action could arise. One family of protein serine/threonine kinases in particular, referred to as microtubule-associated protein kinases or mitogen-activated protein kinases (MAP kinases) or extracellular signal-regulated kinases (ERKs), require phosphorylation on both tyrosine and threonine for enzymatic activation (Anderson *et al.*, 1990; Boulton *et al.*, 1991; Cobb *et al.*, 1991; Payne *et al.*, 1991; Rossomando *et al.*, 1991). Thus the MAP kinases represent a component of the protein kinase cascades through which the protein tyrosine phosphorylation signal elicited by growth factor receptors can be converted to protein serine/threonine-phosphorylation events. Analysis of growth factor-regulated serine/threonine kinase signals indicates that all growth factors (PDGF-BB, NGF, basic FGF, EGF, and IGF-I) for which receptors are expressed on PC12 cells can stimulate two MAP kinase activities. We show in this report that persistent activation of these MAP kinases was observed only by those growth factor receptors that mediate neuronal differentiation (PDGF-BB, basic FGF, and NGF).

MATERIALS AND METHODS

Materials

Human recombinant forms of PDGF-BB, basic FGF, EGF, and IGF-I, as well as the monoclonal antiphosphotyrosine antibody, were purchased from Upstate Biotechnology (Lake Placid, NY). NGF was purified from mouse submaxillary glands by published protocols (Mobley *et al.*, 1976) and was demonstrated to be free of any contaminating EGF. All peptide substrates and inhibitors were synthesized by the peptide synthesis facility at the National Jewish Center and purified by reverse phase high-pressure liquid chromatography. Myelin basic protein was purchased from Sigma Chemical (St. Louis, MO).

Retroviral-Mediated Gene Transfection

The retrovirus packaging cell line, GP+E-86 (Markowitz *et al.*, 1988), was transfected by electroporation with the LXSN retroviral expression vector (Miller and Rosman, 1989) into which the 5.2-kb murine β-PDGF receptor cDNA (a generous gift from Dr. Lewis T. Williams, UC San Francisco) had been inserted at the unique *Eco*RI site. A stable virus-producing GP+E-86 clone was isolated, and growth medium into which the β-PDGF receptor-transfected clone had secreted virus for ~16 h was supplemented with polybrene (8 μg/ml) and then incubated with PC12 cells for 16 h. Similarly, PC12 cells were infected with retrovirus produced by a GP+E-86 clone that had been transfected with the pMV-7 retroviral expression vector (Kirschmeier *et al.*, 1988), lacking a cDNA insert as a control for neo-resistance selection. The virus-infected PC12 cells were seeded onto poly-L-lysine-coated dishes and selected for resistance to G-418 (250 μg/ml) in regular serum-containing growth medium Dulbecco's modified Eagle's medium ([DMEM] containing 5% horse serum and 5% fetal bovine serum) or in plasma-derived serum-containing growth medium (DMEM containing 5% plasma-derived horse serum and 5% plasma-derived newborn calf serum from Cocalico Biologicals, Reamstown, PA). Three to four weeks later, colonies resistant to G-418 were picked and then routinely propagated in plasma-derived serum-containing medium that lacked G-418. The results described in this report are representative of wild-type PC12 cells, two independent PC-Neo clones, and four independent PC-PDGFR clones.

Immunoblot Analyses

For immunoblot analysis of plasmid-expressed β-PDGF receptors in the PC12 clones, plasma membrane fractions of the PC-PDGFR clones were dissolved in reducing sodium dodecyl sulfate (SDS) sample buffer (2% sodium dodecyl sulfate, 5% glycerol, 62.5 mM tris(hydroxymethyl)aminomethane [Tris]-Cl (pH 6.8), 5% β-mercaptoethanol, 0.001% bromphenol blue), resolved by 7.5% SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to nitrocellulose. The filter was blocked with 5% bovine serum albumin and 0.1% Tween-20 in Tris-buffered saline (pH 7.4) and probed with a 1:500 dilution of a rabbit anti-PDGF receptor antiserum (#538, kindly provided by Dr. Tom Daniels, Vanderbilt University). The filter was washed, and bound antibodies were detected with [^{125}I] protein A. For phosphotyrosine immunoblot analysis of Mono Q fast protein liquid chromatography (FPLC) fractions, 900 μl of the fractions were mixed for 18 h at 4°C with 100 μl of 72% trichloroacetic acid and 0.15% sodium deoxycholate. The precipitated proteins were collected by microcentrifugation and washed twice with acetone. The pelleted proteins were resolved on 8% SDS-PAGE gels and transferred to nitrocellulose. The filters were blocked with 3% Cohn-crystallized bovine serum albumin (ICN Biomedicals, Costa Mesa, CA) in Tris-buffered saline and probed with monoclonal antiphosphotyrosine antibodies which were visualized by [^{125}I] protein A.

Mono Q FPLC

Anion exchange chromatography of cell extracts was performed at 4°C with a Pharmacia LKB Biotechnology FPLC system and a Mono Q HR5/5 column. Soluble extracts were prepared from two 10-cm dishes of cells per treatment by collecting the attached cells into 600 μl of 50 mM β-glycerophosphate (pH 7.2), 100 μM sodium vanadate, 2 mM $MgCl_2$, 1 mM ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*,tetraacetic acid (EGTA), 0.5% Triton X-100, 2 μg/ml leupeptin, 2 μg/ml aprotinin, and 1 mM dithiothreitol. After centrifugation in a microcentrifuge for 5 min, soluble extracts (0.5 ml, 0.5–1 mg protein) were applied to a Mono Q FPLC column equilibrated in 50 mM β-glycerophosphate (pH 7.2), 100 μM sodium vanadate, 1 mM EGTA, and 1 mM dithiothreitol. The column was washed with 3 ml of buffer and then bound proteins were eluted with a 30-ml linear 0–0.4 M NaCl gradient in the equilibration buffer. Aliquots (20 μl) of 1-ml fractions were mixed with 20 μl of 50 mM β-glycerophosphate (pH 7.2), 100 μM sodium vanadate, 20 mM $MgCl_2$, 200 μM [γ-^{32}]ATP (2000–5000 cpm/pmol), 50 μg/ml IP-20 (TTYADFIASGRTGRRNAIHD), 1 mM EGTA, and either 400 μM $EGFR_{662-681}$ peptide (RRELVEPLTPSGEAPNQALLR, Takishima *et al.*, 1991) or 0.66 mg/ml myelin basic protein. After a 15-min incubation (30°C), 10 μl of 25% trichloroacetic acid was added (10 μl of 250 mM EDTA for reactions containing myelin basic protein), and the phosphorylated substrates were quantified as described (Heasley and Johnson, 1989a,b).

RESULTS

PDGF Mediates Reversible Differentiation of PC12 Clones Expressing β-PDGF Receptors

Insertion of the mouse β-PDGF receptor cDNA into the retroviral expression vector, LXSN (Miller and Rosman,

1989), followed by packaging into infectious retroviruses in GP+E-86 cells (Markowitz *et al.*, 1988) permitted high-efficiency introduction and expression of the β-PDGF receptor cDNA in PC12 cells. Inspection of PC12 cell cultures, 7 d after retroviral infection and before G-418-mediated killing of nontransfected cells had occurred, revealed a significant number of neuronally differentiated cells when the selection was performed in growth medium containing normal levels of horse (5%) and fetal bovine serum (5%). Three to four wk later when only G-418-resistant colonies remained on the culture dish, numerous colonies selected in medium containing normal serum exhibited a differentiated phenotype. This morphologic differentiation appeared to be due to stimulation of the transfected β-PDGF receptor by PDGF present in the serum because G-418 selection in growth medium containing plasma-derived serum, which is low in PDGF, failed to yield PC12 cells with the differentiated phenotype either at early (7 d) or later culture times.

A key feature of the PC12 differentiation program elicited by NGF and FGF is the complete reversibility after removal of the growth factor from the medium (Greene and Tischler, 1976; Rydel and Greene, 1987). The action of PDGF present in serum to differentiate PC12 cells expressing transfected β-PDGF receptors was reversible. Replacement of the serum-containing medium on the differentiated β-PDGF receptor-transfected PC12 clones with medium containing plasma-derived serum was accompanied by the withdrawal of their neurites, permitting putative β-PDGF receptor-expressing cells to be clonally expanded and propagated for biochemical analysis. In addition, clones that had been selected for G-418 resistance in plasma-derived serum were also randomly picked and screened for the expression of the β-PDGF receptor polypeptide. Immunoblot analysis of the β-PDGF receptor in a PC12 clone selected in plasma-derived serum (PC-PDGFR-111), as well as a clone that had exhibited a differentiated phenotype during selection in normal serum (PC-PDGFR-102) is shown in Figure 1, along with a PC12 clone transfected with a retroviral vector lacking a cDNA insert (PC-Neo-7.2). Note that PC-Neo-7.2 lacks detectable β-PDGF receptor whereas PC-PDGFR-102 and -111 express an immunoreactive β-PDGF receptor polypeptide of ~190–200 kDa.

When cultured in growth medium containing plasma-derived serum or in very low levels of fetal bovine serum (0.3%), the PC-PDGFR clones exhibited a morphology similar to that of parental PC12 cells (Figure 2). Within several days of culture in the presence of human recombinant PDGF-BB, the cells neuronally differentiated as indicated by the extension of long neurites (Figure 2). The PDGF-stimulated morphologic differentiation of PC-PDGFR-102 and -111 cells was as extensive as that achieved with either basic FGF or NGF (Figure 2) and was equally reversible. As with parental PC12 cells,

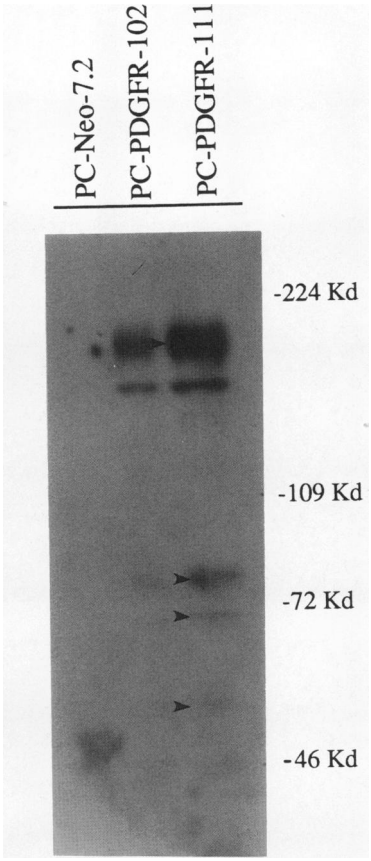

Figure 1. Immunoblot analysis of β-PDGF receptors in transfected PC12 clones. Membrane protein (100 μg) from two PC12 clones stably infected with the LXSN-PDGFR retrovirus (PC-PDGFR-102 and -111) or from a PC12 clone transfected with the expression vector lacking a cDNA insert (PC-Neo-7.2) were dissolved in SDS sample buffer, resolved on a 7.5% polyacrylamide SDS gel, transferred to nitrocellulose, and probed for the cytoplasmic domain of the murine β-PDGF receptor. The full-length receptor polypeptide (~200 kDa) and several putative proteolytic fragments are indicated with arrows.

EGF (Figure 2) and IGF-I failed to direct neuronal differentiation even though their respective receptors are expressed at functional levels on PC12 cells (Dahmer *et al.*, 1989; Dahmer and Perlman, 1988; Huff *et al.*, 1981). Thus unlike other exogenous gene products that differentiate PC12 cells such as v-Src and v-Ras, the βPDGF receptor induces growth factor-regulated and reversible neuronal differentiation of PC12 cells.

Characterization of 43- and 41-kDa Tyrosine-Phosphorylated Polypeptides in Growth Factor-Treated PC-PDGFR Clones as Activated MAP Kinases

MAP kinases, also known as ERKs (Boulton *et al.*, 1991), are a family of protein serine/threonine kinases that have the notable feature of being phosphorylated on tyrosine and threonine residues when purified in their

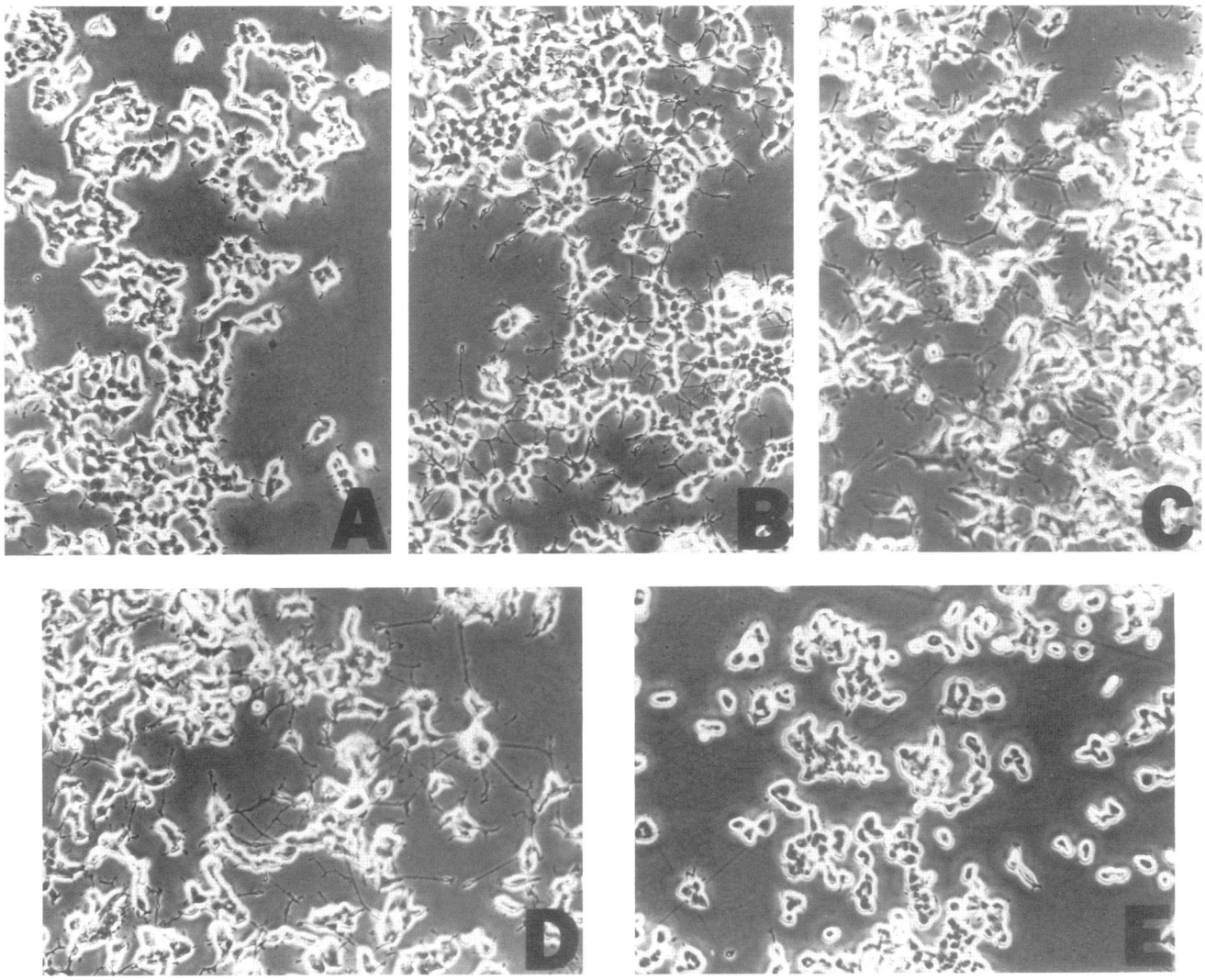

Figure 2. Neuronal differentiation of PC12 cells expressing the β-PDGF receptor in response to PDGF-BB, basic FGF, and NGF. PC-PDGFR-102 cells were cultured for 5 d in DMEM containing 0.3% fetal bovine serum alone (E) or medium supplemented with 10 ng/ml EGF (A), 30 ng/ml basic FGF (B), 100 ng/ml NGF (C), or 30 ng/ml PDGF-BB (D). The morphological differentiation response exhibited by this PC-PDGFR clone is representative of three other independent clones.

activated forms (Anderson *et al.*, 1990; Payne *et al.*, 1991). Because of this characteristic, these protein kinases are thought to serve, in part, as the switching mechanism between the activated receptor tyrosine kinases and the protein serine/threonine kinase cascades that they regulate in cells. Similar to recent reports of MAP kinase activation in fibroblasts (Ahn *et al.*, 1991) and PC12 cells (Boulton *et al.*, 1991; Miyasaka *et al.*, 1991; Schanen-King *et al.*, 1991), we observed highly inducible tyrosine phosphorylation of putative MAP kinase polypeptides after stimulation of either wild-type PC12 cells or the PC-PDGFR clones with NGF, basic FGF, PDGF-BB, EGF, and IGF-I. To assess the activity of MAP kinases in PC-PDGFR cells after incubation with various growth factors, extracts from PC-PDGFR cells treated with or without growth factors were fractionated by Mono Q FPLC, and the fractions were assayed for MAP kinase. Figure 3A shows that the majority of the $EGFR_{662-681}$ peptide kinase activity from cells treated for 10 min with PDGF-BB resolved into two peaks centered at fraction 14 (0.16 M NaCl) and 17 (0.2 M NaCl) with a minor peak of activity eluting in the flow-through fractions (fractions 1–6). Identical chromatographic profiles were observed with NGF and basic FGF as well as with EGF (Fig. 3A) and IGF-I. The growth factor-stimulated increase in fractionated MAP kinase activity compared with activity present in fractions from untreated control cells was ~2- to 3-fold for the flow through fractions and greater than 30-fold in the peaks eluting at 0.16 and 0.2 M NaCl.

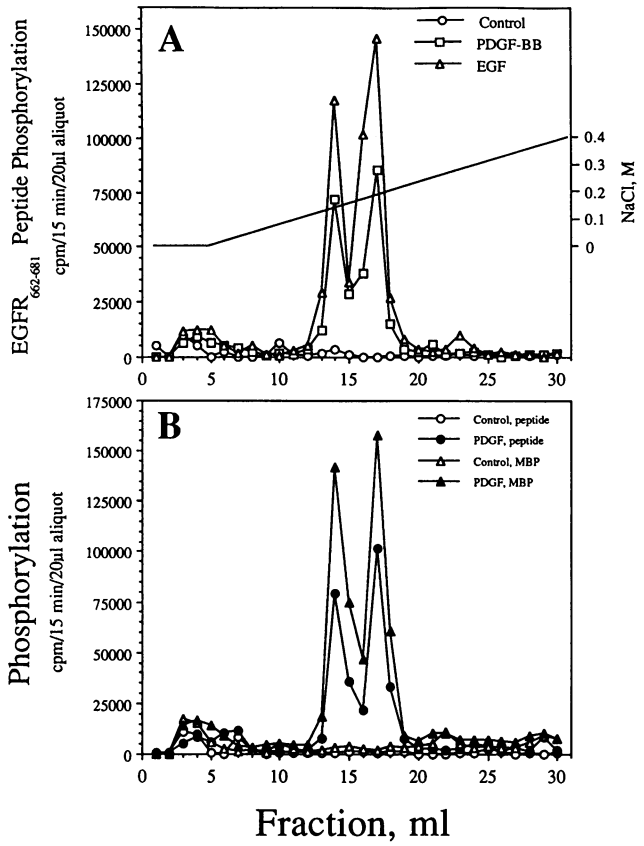

Figure 3. Anion exchange chromatography of growth factor-stimulated MAP kinases in PC-PDGFR cells. PC-PDGFR-111 cells (two 10-cm dishes per treatment) cultured for 16 h in DMEM containing 0.1% bovine serum albumin were incubated for 10 min with the indicated growth factors, rinsed with ice-cold phosphate-buffered saline, and lysed in 0.6-ml lysis buffer (see MATERIALS AND METHODS). Aliquots (20 µl) of the Mono Q-fractionated extracts were assayed (15 min, 30°C) for MAP kinase activity with $EGFR_{662-681}$ peptide (A and B) or myelin basic protein (B). The data in A and B are from different experiments.

The growth factor-regulated MAP kinase activity that eluted from the Mono Q FPLC column at 0.16 and 0.2 M NaCl coeluted with growth factor-stimulated myelin basic protein (MBP) kinase activity (Fig. 3B) where myelin basic protein is an excellent in vitro substrate for MAP kinase (Erickson *et al.*, 1990). The kinase assay buffer contains inhibitors that exclude the activities of other protein kinases known to phosphorylate myelin basic protein (cAMP-dependent protein kinase, protein kinase C, Ca^{++}/calmodulin-dependent kinases). It is noteworthy that growth factor-stimulated MBP kinase activity in Swiss 3T3 cells and PC12 cells also resolves into two peaks on Mono Q FPLC (Ahn *et al.*, 1990, 1991; Boulton *et al.*, 1991; Miyasaka *et al.*, 1991). Thus the MAP kinase activities stimulated by growth factors in PC-PDGFR cells exhibit chromatographic properties similar to previously described MAP kinases.

The growth factor-regulated MAP kinases eluting at 0.16 and 0.2 M NaCl are related to two phosphotyrosine-containing polypeptides of ~41 and 43 kDa as evidenced by their strict coelution on Mono Q FPLC (Figure 4). The molecular weight of the polypeptides as indicated by SDS-PAGE and phosphotyrosine immunoblotting is consistent with the apparent molecular size of the $EGFR_{662-681}$ peptide-detected MAP kinase activity (35–50 kDa) as estimated by Superose gel exclusion FPLC, as well as MAP kinase and MBP kinase

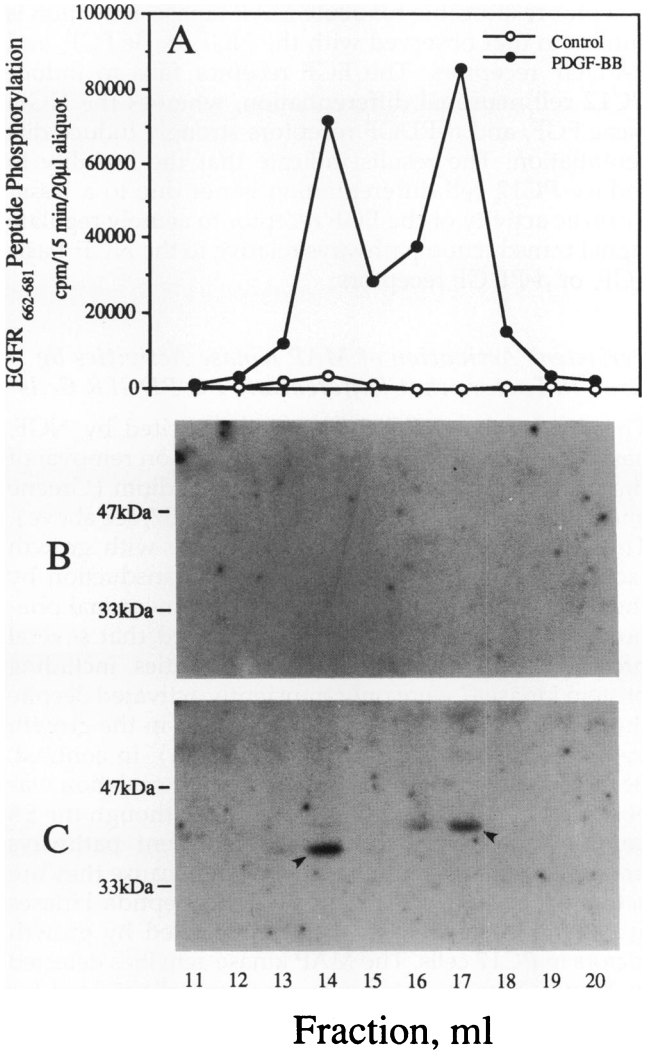

Figure 4. Coelution of $EGFR_{662-681}$ peptide-detected MAP kinase activities and tyrosine-phosphorylated polypeptides of 41 and 43 kDa on anion-exchange columns. PC-PDGFR-111 cells (two 10-cm dishes per treatment) were incubated (37°C) for 10 min with or without 30 ng/ml PDGF-BB and soluble extracts were prepared and chromatographed on Mono Q FPLC as described in MATERIALS AND METHODS. Aliquots (20 µl) of each fraction were assayed for MAP kinase activity with the $EGFR_{662-681}$ peptide (A) or for phosphotyrosine content by immunoblotting (see MATERIALS AND METHODS). Autoradiographs of immunoblots from control (B) and PDGF-treated samples (C) are shown.

activities observed in Swiss 3T3 cells and PC12 cells (Ahn *et al.*, 1990; Schanen-King *et al.*, 1991). Thus the MAP kinase activities stimulated by growth factors in PC-PDGFR clones behave identically to previously described MAP kinases and correlate with two phosphotyrosine-containing polypeptides of 41 and 43 kDa that represent the catalytic MAP kinase activities.

Acute exposure (5 min) of PC12 cells to EGF results in similar activation and phosphorylation of MAP kinases as that observed with NGF (Gotoh *et al.*, 1990; Miyasaka *et al.*, 1991) or PDGF (Figures 3 and 4). These findings indicate that the intrinsic activity of the EGF receptor responsible for acute MAP kinase activation is similar to that observed with the NGF, basic FGF, and β-PDGF receptors. The EGF receptor fails to induce PC12 cell neuronal differentiation, whereas the NGF, basic FGF, and β-PDGF receptors strongly induce differentiation. The results indicate that the inability to induce PC12 cell differentiation is not due to a lesser intrinsic activity of the EGF receptor to acutely regulate signal transduction pathways relative to the NGF, basic FGF, or β-PDGF receptors.

Persistent Activation of MAP Kinase Activities by Growth Factors that Differentiate PC-PDGFR Cells

The differentiated PC12 phenotype elicited by NGF, basic FGF, and PDGF is readily reversed on removal of the growth factors from the culture medium (Greene and Tischler, 1976; Rydel and Greene, 1987; see above). The requirement for continuous culture with growth factor indicates that persistent signal transduction by the receptors is required to maintain the neuronal phenotype. In a previous study, we showed that several protein serine/threonine kinase activities including protein kinase C were only transiently activated despite the continuous presence of NGF or EGF in the growth medium (Heasley and Johnson, 1989a,b). In contrast, persistent elevation of S6 peptide phosphorylation was observed in response to NGF or EGF. Although the S6 peptide kinases are unlikely to represent pathways unique to the differentiation program because they are activated by both NGF and EGF, S6 peptide kinases are examples of persistent signals elicited by growth factors in PC12 cells. The MAP kinase activities detected in Mono Q-fractionated extracts from cells treated for 24 h with NGF, PDGF-BB, or basic FGF (Fig. 5A) were elevated 10-fold over control. In contrast, the level of MAP kinase activity in fractionated extracts from PC-PDGFR cells treated for 24 h with EGF (Fig. 5A) or IGF-I was only marginally elevated over control (~2-fold). The EGF and IGF-I-stimulated MAP kinase returned to near basal levels within 3–5 h of the initial stimulation. Coincident with the persistent increase of MAP kinase activity observed in fractionated extracts from NGF, basic FGF, and PDGF-BB-treated cells (Fig. 5A) was the increased phosphotyrosine content of the 43- and 41-kDa MAP kinase polypeptides in fractionated extracts from NGF, basic FGF, and PDGF-treated cells compared with extracts from control cells and cells treated with EGF (Fig. 5B). Thus, NGF, basic FGF, and PDGF-BB, which stimulate neuronal differentiation of PC12 cells, persistently elevated p43- and p41-MAP kinase phosphorylation and activity whereas EGF and IGF-I caused only transient activation and tyrosine phosphorylation of this protein serine/threonine kinase activity.

DISCUSSION

In this report, we have compared the cellular actions of five growth factors whose receptors are functional ligand-regulated tyrosine kinases expressed in PC12 cells. Receptor tyrosine kinases for NGF, basic FGF, EGF, and IGF-I are endogenous, whereas the β-PDGF receptor was exogenously expressed. Although the five growth factor receptors stimulated tyrosine phosphorylation of 41- and 43-kDa cellular MAP kinase proteins and acutely activated MAP kinases, receptors for NGF, basic FGF, and PDGF provided a persistent elevation of MAP kinase activity compared with EGF and IGF-I receptors. In addition, persistent tyrosine phosphorylation of the 43- and 41-kDa MAP kinases after 24 h of growth factor treatment was specifically observed in response to NGF, basic FGF, and PDGF-BB (Figure 5B). Thus receptor tyrosine kinases that promote neuronal differentiation persistently elevated MAP kinase activity, consistent with the requirement for selective but continuous protein kinase signaling by NGF, FGF, and PDGF receptors to maintain the differentiated phenotype. The fact that EGF and IGF-I, which fail to differentiate PC12 cells, can only transiently activate the same MAP kinase pathways indicates that persistent MAP kinase activation is probably vital to the differentiation response by growth factors.

Recently, a number of other reports have appeared concerning growth factor-regulated MAP kinase activities in PC12 cells (Gomez *et al.*, 1990; Gotoh *et al.*, 1990; Miyasaka *et al.*, 1990; Tsao *et al.*, 1990; Boulton *et al.*, 1991; Miyasaka *et al.*, 1991; Schanen-King *et al.*, 1991). In general, the findings of these reports are supportive of our results with respect to chromatographic properties and molecular size. However, kinetics of activation beyond 3 h were not investigated, although Gotoh *et al.* (1990) showed a slower rate of decay in MAP kinase activity after NGF-treatment relative to EGF at early time points. Similar to our findings, Tsao *et al.* (1990) observed selective activation of a protein kinase in PC12 cells by NGF and FGF that phosphorylated high molecular weight MAPs. In these studies, however, NGF and FGF elevated MAP kinase activity 15–20-fold whereas EGF, insulin, and TPA were poor activators even after relatively short treatments. Thus the kinetics of MAP kinase activation reported by Tsao

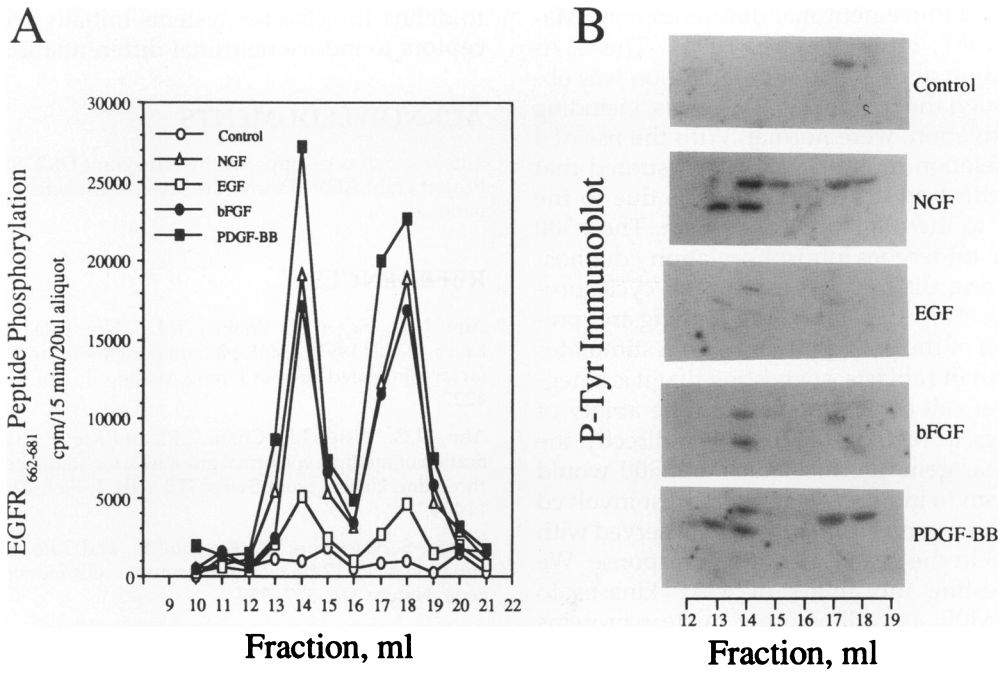

Figure 5. Persistent activation and tyrosine phosphorylation of MAP kinases in PC-PDGFR cells incubated for 24 h with NGF, basic FGF, or PDGF-BB. PC-PDGFR-111 cells (two 10-cm dishes per treatment) were incubated for 24 h with medium alone, 100 ng/ml NGF, 30 ng/ml PDGF-BB, 30 ng/ml basic FGF, or 10 ng/ml EGF and lysed at 4°C in 600 μl of lysis buffer. Centrifuged extracts (0.5 ml, 1 mg protein) were fractionated on Mono Q FPLC as described in MATERIALS AND METHODS. (A) Column fractions were assayed for $EGFR_{662-681}$ peptide phosphorylation. (B) The proteins in the indicated column fractions were precipitated with trichloroacetic acid and immunoblotted with anti-phosphotyrosine antibody as described in MATERIALS AND METHODS. Phosphotyrosine content of MAP kinase polypeptides qualitatively, but not quantitatively, correlates with enzyme activity determined in A. This difficulty in quantitative correlation has been previously observed (Cobb *et al.*, 1991; Rossomando *et al.*, 1991). However, the phosphotyrosine content and activity of MAP kinase is reproducibly and significantly greater in extracts from PDGF, NGF, and basic FGF-treated cells compared with control or EGF-treated cells and was observed in three independent experiments. The somewhat lower phosphotyrosine content of the MAP kinase polypeptides from the basic FGF-treated cells relative to NGF- and PDGF-treated cells was related to incomplete transfer to nitrocellulose in the corresponding region of the SDS gel and was not observed in two other independent experiments.

et al. (1990) is significantly different from our results where we observe marked activation of MAP kinase activities after short exposures (10 min) to all growth factors, but persistent activation only by NGF, basic FGF, and PDGF-BB. Significant differences exist in chromatography systems, cell lysis buffers, and protein kinase substrates so that further investigation will be required for accurate comparison of our study to that of Tsao *et al.* (1990).

MAP kinases and their kinetics of activation have been largely defined in mitogen-stimulated fibroblasts where transient activation during the G0-G1 transition is observed (Ahn *et al.*, 1990). The observations in fibroblasts are similar to the transient activation of MAP kinases we measure in PC12 cells treated with EGF or IGF-I, where EGF and IGF-I are established mitogens (Huff *et al.*, 1981; Dahmer and Perlman, 1988). In contrast to mitogenic stimuli, NGF causes PC12 cells to arrest in G1 during differentiation (Rudkin *et al.*, 1989). A similar G1 arrest appears to occur with basic FGF and PDGF-BB in PC-PDGFR clones (Heasley and Johnson, unpublished observations). Thus, a role for persistently elevated MAP kinase activity would be predicted in response to differentiating agents if MAP kinases are involved in controlling progression through G1. In fact, there is precedent for a role of MAP kinase-like enzymes controlling cell cycle progression in the yeast, *Saccharomyces cerevisiae*, where two protein kinases, referred to as FUS3 (Elion *et al.*, 1990) and KSS1 (Courchesne *et al.*, 1989) are ~50% identical to the cloned mammalian MAP kinases (Boulton *et al.*, 1991) and mediate the response to pheromones. Specifically, the FUS3-encoded protein kinase is required for pheromone-induced cell cycle arrest in G1 (Elion *et al.*, 1990) whereas the KSS1 protein kinase promotes reentry into the cell cycle after pheromone-induced cell cycle arrest (Courchesne *et al.*, 1989). The persistent MAP kinase activity in response to NGF, basic FGF, and PDGF-BB may function in arresting PC12 cells in G1 during neuronal differentiation analogous to the role of FUS3 in the yeast pheromone response.

We have previously demonstrated that perturbation of normal cell cycle control in PC12 cells by expression of adenovirus E1A proteins inhibits the ability of NGF

and basic FGF to induce neuronal differentiation (Maruyama *et al.*, 1987; Heasley *et al.*, 1991). The E1A-mediated inhibition of neuronal differentiation was observed even though the protein kinase signals, including MAP kinase activation, were normal. With the use of a series of E1A deletion mutants, we demonstrated that the inhibitory effect on differentiation was due to the binding of E1A to the p300 nuclear protein. The p300 nuclear protein undergoes phosphorylation/dephosphorylation during different stages of cell cycle progression (Yaciuk *et al.*, 1991), and the binding and presumed inhibition of the p300 protein by E1A stimulates DNA synthesis in fibroblasts, suggesting that it is a negative regulator of cell cycle progression. The ability of the MAP kinases to regulate directly or indirectly the activity of nuclear gene products such as p300 would be one mechanism to induce G1-growth arrest involved in PC12 cell differentiation, similar to that observed with the role of FUS3 in the yeast pheromone response. We are presently testing the ability of MAP kinases to phosphorylate p300, as well as other nuclear proteins that might be involved in cell cycle and differentiation control.

NGF, basic FGF, and PDGF-BB stimulate neuronal differentiation of PC12 cells and persistently elevate MAP kinase activity by interaction with specific receptor tyrosine kinases. However, the features of these receptor kinases that are lacking in the EGF and IGF-I receptors that account for their selective signaling functions remain undefined. One possibility is that profound downregulation of the EGF and IGF-I receptors occurs relative to NGF, basic FGF, and PDGF receptors such that a significant level of persistent signaling by EGF or IGF-I is not achieved. However, the fact that both EGF and NGF can maintain for $\geq$24 h an equivalent activation state of S6 peptide kinases in PC12 cells (Heasley and Johnson, 1989a) argues against selective downregulation of the EGF or IGF-I responses as the sole mechanism for their failure to persistently activate MAP kinases or direct PC12 cell differentiation. Instead, a selective loss of EGF and IGF-I receptor signaling, possibly by feedback regulation of the receptors, seems more likely. The EGF receptor is unable to direct PC12 cell differentiation even when significantly overexpressed (Yan *et al.*, 1991), indicating that the differentiation response is driven by specific tyrosine kinases. This infers that it is the selective regulation of specific gene products such as PLCγ, PI3 kinase, and GAP by different tyrosine kinase encoded receptors (Lev *et al.*, 1991) that initiates and maintains the differentiation response. Our finding that transfected PDGF receptors mimic the endogenous NGF and FGF receptors permits, for the first time, a genetic approach for defining the effectors necessary for reversible, growth factor-mediated PC12 cell differentiation. Wild-type and mutant PDGF receptors (Kazlauskas *et al.*, 1991) can be used to define the effector systems initially regulated by receptors to induce neuronal differentiation.

ACKNOWLEDGMENTS

This research was supported by NIH grant DK37871, NIH Program Project grant AI29903, and the Juvenile Diabetes Foundation, International.

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