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Mitogen-Activated Protein Kinase Activation Is Insufficient for Growth Factor Receptor-Mediated PC12 Cell Differentiation

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When expressed in PC12 cells, the platelet-derived growth factor β receptor (βPDGF-R) mediates cell differentiation. Mutational analysis of the βPDGF-R indicated that persistent receptor stimulation of the Ras/Raf/mitogen-activated protein (MAP) kinase pathway alone was insufficient to sustain PC12 cell differentiation. PDGF receptor activation of signal pathways involving p60$^{c\text{-}src}$ or the persistent regulation of phospholipase Cγ was required for PC12 cell differentiation. βPDGF-R regulation of phosphatidylinositol 3-kinase, the GTPase-activating protein of Ras, and the tyrosine phosphatase, Syp, was not required for PC12 cell differentiation. In contrast to overexpression of oncoproteins involved in regulating the MAP kinase pathway, growth factor receptor-mediated differentiation of PC12 cells requires the integration of other signals with the Ras/Raf/MAP kinase pathway.

The platelet-derived growth factor receptor (PDGF-R) is a transmembrane polypeptide encoding an intrinsic tyrosine kinase in its intracellular domain. Two distinct PDGF-R genes encode either an α (7, 42, 47) or a β (6, 19, 73) subunit. Binding of PDGF (22) induces dimerization (23) and *trans* phosphorylation of the βPDGF-R on specific tyrosine residues (35). The phosphorylated receptor initiates a series of intracellular signals which ultimately lead to cell growth (12), differentiation (20), and chemotaxis (70) depending on the cellular context.

A number of tyrosines on the intracellular domain of the βPDGF-R are phosphorylated upon activation of the receptor and serve as recognition sites for proteins which contain Src homology 2 (SH2) domains (57). For example, the βPDGF-R has been shown to associate with Src family tyrosine kinases p60$^{c\text{-}src}$, p59^{fyn}, and p62$^{c\text{-}yes}$ (39) via juxtamembrane tyrosines 579 and 581, which are in vivo phosphorylation sites (50). The SH2 domain-containing proteins p46 and p52 (Shc proteins) bind to the βPDGF-R at multiple phosphotyrosines including tyrosine 581 and indirectly via association with other tyrosine-phosphorylated proteins (74). Phosphorylation of tyrosines 740 and 751 is critical for association of the p85 regulatory subunit of phosphatidylinositol 3-kinase (PI3-K) with the βPDGF-R (2, 8, 29–31). Phosphorylated tyrosine 751 also binds Nck via its SH2 binding domain (52). The GTPase-activating protein (GAP) of Ras is tyrosine phosphorylated in response to PDGF and binds to the receptor at phosphorylated Tyr-771 (27, 33, 49). The SH2-containing phosphotyrosine phosphatase, Syp, associates with phosphorylated tyrosine 1009 (34, 41). In addition, there is evidence which suggests that the adaptor protein, Grb2, associates with the βPDGF-R via tyrosine 716 (1) and indirectly through Syp (44). Phosphorylated tyrosine 1009 may also influence the binding of phospholipase Cγ (PLCγ) to tyrosine 1021 (28, 59). Mutational analysis of the βPDGF-R has demonstrated that phosphorylated tyrosines 740 and 751, which mediate association with PI3-K, and tyrosine 1021, which mediates association with PLCγ, are necessary for the transduction of PDGF-dependent mitogenic signals (68). Mutation of Tyr-857, a major autophosphorylation site, decreased intrinsic βPDGF-R tyrosine kinase activity and tyrosine phosphorylation of several substrate proteins (15, 32).

In PC12 cells, cellular differentiation is mediated by nerve growth factor (NGF) and its cognate tyrosine kinase receptor, Trk (see reference 5 for a review). The βPDGF-R, like the NGF receptor, mediates cellular differentiation when expressed in PC12 cells (20). Neuronal differentiation that is mediated by tyrosine kinase receptors for NGF and PDGF is characterized by neurite outgrowth and cessation of growth (18), induction of sodium channels (13), persistent activation of mitogen-activated protein (MAP) kinase (20), and PLCγ (58). Overexpression of oncoproteins such as p60$^{c\text{-}src}$, p21^{ras}, p74^{raf}, and MEK also mediates neurite outgrowth in PC12 cells and demonstrates a requirement for MAP kinase activation for neuronal differentiation (3, 10, 38, 61, 62, 71, 72). Unfortunately, overexpression of oncoproteins does not mimic or faithfully represent regulation of signal pathways. Rather, overexpression of these proteins may strongly drive pathways constitutively and alter cellular phenotype differently than normal receptor regulation of cellular response.

Mutational analysis of Trk has defined critical tyrosines that specifically regulate the activities of PLCγ, PI3-K, and Shc (45, 54–56, 60). PLCγ and Shc regulation appear to play a major role for NGF-mediated neurite outgrowth (54, 60), whereas PI3-K activity is not required (54). The βPDGF-R regulates a greater number of signaling events than that characterized for Trk. Therefore, mutational analysis of βPDGF-R signaling provides a method to determine combinations of different receptor-mediated signal pathways which control PC12 cell differentiation without activation of the Trk receptor. In this report, we demonstrate that specific combinations of intracellular signals are necessary for growth factor receptor-mediated PC12 cell neuronal differentiation.

MATERIALS AND METHODS

Site-directed mutagenesis. The *Sph*I-*Bam*HI 1.35-kb fragment of the human βPDGF-R was subcloned into the pAlter-1 vector and subjected to site-directed

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mutagenesis by using the Promega Altered Sites In Vitro Mutagenesis System. To change Tyr-579 to Phe, the antisense oligonucleotide 5′-GTC CACGTAGATGA*ACTCATGGCCGTC-3′ was used. The asterisk indicates a change from the wild-type (WT) DNA sequence. To change Tyr-579 and -581 to Phe, the antisense oligonucleotide 5′-CATGGGGTCCACGA*AGATGA*A CTCATGGCCGTC-3′ was used. To produce a Tyr-to-Phe mutation at position 581, the antisense oligonucleotide 5′-GGGGTCCACA*A*AGATGTACTC ATGA*CCGTCAGAGCT-3′, which introduced a *Bsp*HI site without affecting the amino acid sequence, was used with the Amersham Oligonucleotide-Directed In Vitro Mutagenesis System (30). All mutations were verified by dideoxy DNA sequencing and by restriction digest, when possible. The 1.35-kb *Sph*I-*Bam*HI fragments containing F-579, F-581, and F-579/81 were subcloned into RR7 (67). The *Eco*RI-*Sal*I 4.2-kb fragment of RR7, which encodes the entire human βPDGF-R, was subcloned into the *Eco*RI-*Sal*I sites of pLXSN[2], which has a polylinker modified from that of pLXSN consisting of *Eco*RI, *Not*I, *Hpa*I, *Sal*I, and *Bam*HI.

The F5 βPDGF-R construct consists of five Tyr-to-Phe mutations at positions 740, 751, 771, 1009, and 1021 (68). The entire βPDGF-R construct which contained these mutations was termed RR3. The *Sph*I-*Sac*II 0.47-kb fragments containing F-579, F-581, and F-579/81 were subcloned into the *Sph*I-*Sac*II sites of the F5 βPDGF-R in RR3. These βPDGF-R constructs were subcloned into pLXSN[2] as described above and are referred to as F5/579, F5/581, and F5/579/81, respectively (see Fig. 1). The kinase-inactive βPDGF-R, K634R, was prepared as described previously (30). The plasmids were propagated in *Escherichia coli* JM109 and then purified by column chromatography according to the protocols of Qiagen, Inc. (Chatsworth, Calif.).

PDGF-R expression. Plasmids that contain βPDGF-R constructs were introduced as calcium phosphate precipitates into an NIH 3T3 packaging cell line, Ψ2, as previously described (46). After 48 h, a second NIH 3T3 packaging cell line, PA317, was infected with the ecotropic virus which was produced by the Ψ2 cells. The PA317 cells were cultured in the presence of 0.5 mg of G418 per ml 24 h postinfection. Drug-resistant cells were pooled and propagated for several passages. Amphotrophic retrovirus was collected from subconfluent cultures of PA317 cells, and proliferating PC12 cells on a 10-cm dish were infected with 3 ml of viral supernatant supplemented with 4 μg of Polybrene per ml. After 1 h, 5 ml of medium containing plasma-derived serum, described below, was supplemented with 4 μg of Polybrene per ml and added to the PC12 cells. After 24 h, the viral supernatant was replaced with medium containing plasma-derived serum, and the cells were allowed to grow for another 24 h. Infected cells were then selected over a 2-week period in 1 mg of G418 per ml. Clones were propagated and screened for βPDGF-R expression by immunoblotting. The cell lysates were prepared by washing twice with ice-cold phosphate-buffered saline and lysing with 0.5 ml of lysis buffer consisting of 70 mM β-glycerophosphate (pH 7.2), 100 μM Na_3VO_4, 2 mM $MgCl_2$, 1 mM EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid], 0.5% Triton X-100, 5 μg of leupeptin per ml, 20 μg of aprotinin per ml, and 1 mM dithiothreitol. Nuclei and cell debris were removed by centrifugation at 14,000 × *g* for 10 min. The supernatants were normalized for protein by the Bradford method (4) with bovine serum albumin (BSA) as the standard.

Cell culture. PC12 cells were maintained in Dulbecco's modified Eagle's medium with 5% plasma-derived horse serum and 5% plasma-derived newborn calf serum (Cocalico Biologicals, Reamstown, Pa.) supplemented with 100 μg 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% CO_2.

Neurite outgrowth assay. PC12 cells were seeded on six-well dishes which were coated with rat tail collagen (Upstate Biotechnology, Inc.). After 24 h, the cells were maintained in Dulbecco's modified Eagle's medium supplemented with 1% plasma-derived horse serum, penicillin, and streptomycin and then challenged with either 30 ng of PDGF per ml or 100 ng of NGF per ml (Upstate Biotechnology, Inc.) for a period of 6 days. The medium and growth factors were replaced after 3 days. Differentiation is defined as the extension of neurites that are at least twice the length of the cell body.

MAP kinase assay. The p42 and p44 isoforms of MAP kinase were assayed by using the EGF-$R_{662–681}$ peptide as a phosphorylation substrate (17, 20). Cell extracts were prepared and adsorbed to DEAE-Sephacel columns as described previously (21).

Phospholipase Cγ immunoblotting. PC12 cells expressing the mutant PDGF-Rs were stimulated with PDGF for 10 min at 37°C. The cells were lysed as described above for βPDGF-R expression, and protein content was normalized for each of the lysates. Approximately 1.0 mg of total protein was immunoprecipitated with antiphosphotyrosine antibodies (Upstate Biotechnology, Inc.). Following sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transfer to nitrocellulose, proteins were immunoblotted with monoclonal antibodies raised against PLCγ (Upstate Biotechnology, Inc.). Immunoreactive proteins were visualized with ^{125}I-protein A after treatment with rabbit anti-mouse immunoglobulin G (Cappel).

Ras activation assay. Ras GTP-loading experiments were performed as described previously (66). Briefly, quiescent PC12 cells were radiolabeled with $^{32}PO_4$ for 10 to 12 h and then stimulated with PDGF for 10 min at ambient temperature. Cells were lysed, and Ras was immunoprecipitated with monoclonal antibody Y13-259. The nucleotides were separated by polyethyleneimine cellulose thin-layer chromatography with 0.75 M KH_2PO_4, pH 3.5. The ratio of GTP to GDP which was bound to immunoprecipitated Ras was quantitated by using a PhosphorImager (Molecular Dynamics).

B-Raf and Raf-1 assay. Raf Ser/Thr kinases were assayed, as a measure of MAP kinase activation, as previously described (17, 65). Briefly, quiescent cells were stimulated with growth factors, the cells were lysed, and the protein content was normalized prior to immunoprecipitation (4). The immunoprecipitated Raf proteins were used to phosphorylate recombinant kinase-inactive MEK-1. Proteins were resolved by SDS–10% PAGE, and radiolabeled kinase-inactive MEK-1 was quantitated by using a PhosphorImager.

p60$^{c\text{-}src}$ assay. PC12 cells were deprived of serum for 12 to 18 h with Dulbecco's modified Eagle's medium supplemented with 0.1% BSA, 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) (pH 7.2), 100 μg of streptomycin per ml, and 100 U of penicillin per ml. All PC12 cell stimulations were performed with 30 ng of PDGF-BB per ml for 10 min at 37°C unless stated otherwise. Cell lysates were prepared as described above. The PDGF-R was immunoprecipitated by adding antibody directed against the carboxyl terminus of the receptor (32). After 90 min at 4°C, the immune complexes were incubated with formalin-fixed protein A (Sigma) for 30 min at 4°C. The immune complexes were layered in polystyrene tubes (10 by 75 mm) on 0.6 ml of 10% sucrose in lysis buffer and collected by centrifugation at 2,500 rpm for 20 min. The pellet was washed once with lysis buffer, twice with 1 ml of PAN {10 mM PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)] [pH 7.0], 100 mM NaCl, 20 μg of aprotinin per ml} plus 0.5% Nonidet P-40, and once with PAN and collected by centrifugation for 7 min as described above. The final pellet was washed with 0.5 ml of PAN and collected by centrifugation at 5,000 rpm for 90 s in a screw-cap microcentrifuge tube. The pellet was resuspended in 21 μl of PAN, then 4 μl of kinase buffer (200 mM PIPES [pH 7.0], 100 mM $MnCl_2$, 20 μg of aprotinin per ml) and 15 μl of [γ-^{32}P]ATP (10 μCi/μl, 2,000 to 5,000 dpm/pmol) were added, and the in vitro kinase reaction was allowed to proceed for 15 min at 30°C. The reaction was terminated with 15 μl of 2% SDS and 4 mM dithiothreitol and then heated at 100°C for 3 min. The insoluble protein A was collected by centrifugation for 5 min at 15,000 rpm, and then the supernatant was transferred to 1 ml of radioimmunoprecipitation assay buffer-EDTA (10 mM $NaPO_4$ [pH 7.0], 150 mM NaCl, 2 mM EDTA, 1% sodium deoxycholate, 1% Nonidet P-40, 0.1% SDS, 1% aprotinin, 50 mM NaF, 200 μM Na_3VO_4, 0.1% β-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride) supplemented with 5 μl of Src monoclonal antibody 327 (Oncogene Science). After 90 min at 4°C, 5 μl of 2-mg/ml rabbit anti-mouse immunoglobulin G (Cappel) was added, and the immune complexes were allowed to form for 30 min. Formalin-fixed protein A was added and incubated for an additional 30 min. The immune complexes were collected by centrifugation for 20 min at 2,500 rpm in tubes (10 by 75 mm) on 0.6 ml of 10% sucrose in radioimmunoprecipitation assay buffer-EDTA. The immune complexes were washed twice with 1 ml of radioimmunoprecipitation assay buffer-EDTA, twice with PAN–Nonidet P-40, and once with PAN and collected by centrifugation at 2,500 rpm for 7 min. The final pellet was washed with 0.5 ml of PAN and collected by centrifugation at 5,000 rpm for 90 s. The pellet was resuspended in Laemmli sample buffer and heated at 100°C for 5 min, and then the proteins were resolved by SDS-PAGE (16). The gel was fixed in 50% methanol–10% acetic acid for 1 h and then immersed in 100% methanol for 20 min. In order to reduce nonspecific radioactivity and Ser/Thr phosphorylation, the gel was treated with 1 M KOH for 1 h at 55°C and then fixed as described above with repeated changes of fix solution prior to drying and autoradiography.

RESULTS

Experimental approach. Since a characteristic of PC12 cell differentiation is elevated and persistent MAP kinase activity, our initial strategy for dissecting the signaling pathways involved in PC12 cell differentiation focused on introducing mutant PDGF-Rs which had single tyrosine-to-phenylalanine mutations which would disrupt receptor-mediated activation of MAP kinase. This approach was designed to define selected signaling pathways, in addition to MAP kinase, that are necessary for PC12 cell differentiation. Our studies with single tyrosine mutations in the βPDGF-R failed to define a specific tyrosine autophosphorylation site that, by itself, was critical for mediating PC12 cell differentiation. The only receptor mutation that failed to mediate differentiation was the kinase-inactive receptor, K634R, which indicated that the βPDGF-R tyrosine kinase activity was required for this response. Thus, the results indicated that there were probably multiple, simultaneously activated signaling pathways and that the loss of regulation of these individual pathways had no effect on PC12 cell differentiation.

For this reason, multiple tyrosine-to-phenylalanine mutations were introduced into the βPDGF-R. This approach al-

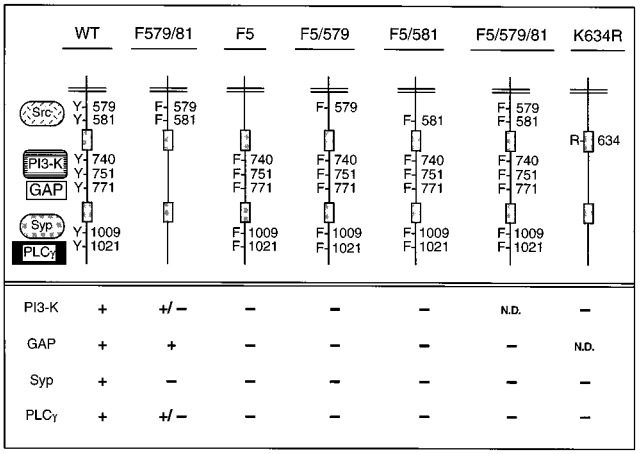

FIG. 1. Schematic diagram of the intracellular domain of the βPDGF-R. The intracellular domain consists of a juxtamembrane domain, two kinase domains (shaded boxes), a kinase insert domain located between the kinase domains, and a carboxyl-terminal domain. The cell membrane is depicted as two horizontal lines, and the extracellular domain is not shown. The WT βPDGF-R contains tyrosines at positions 579, 581, 740, 751, 771, 1009, and 1021 which become phosphorylated upon PDGF stimulation. The phosphorylated tyrosines are recognized by the specific SH2 domains of $p60^{c\text{-}src}$, PI3-K, GAP, Syp, and PLCγ, which results in the association of these proteins with the βPDGF-R. Tyrosine-to-phenylalanine mutations were made by site-directed mutagenesis. The nomenclature that is used to describe the various mutant PDGF-Rs is indicated above the respective receptors. The active-site lysine in the first kinase domain was mutated to arginine and is designated K634R. The regulation of PI3-K, GAP, Syp, and PLCγ by the various mutant PDGF-Rs was measured as previously described (68), and the results are summarized below the respective receptors. A plus sign indicates activation similar to that of the WT PDGF-R, a minus sign indicates no activation, the plus-minus sign indicates that the F579/81 PDGF-R activates PI3-K and PLCγ but not as well as the WT βPDGF-R, and N.D. indicates not determined.

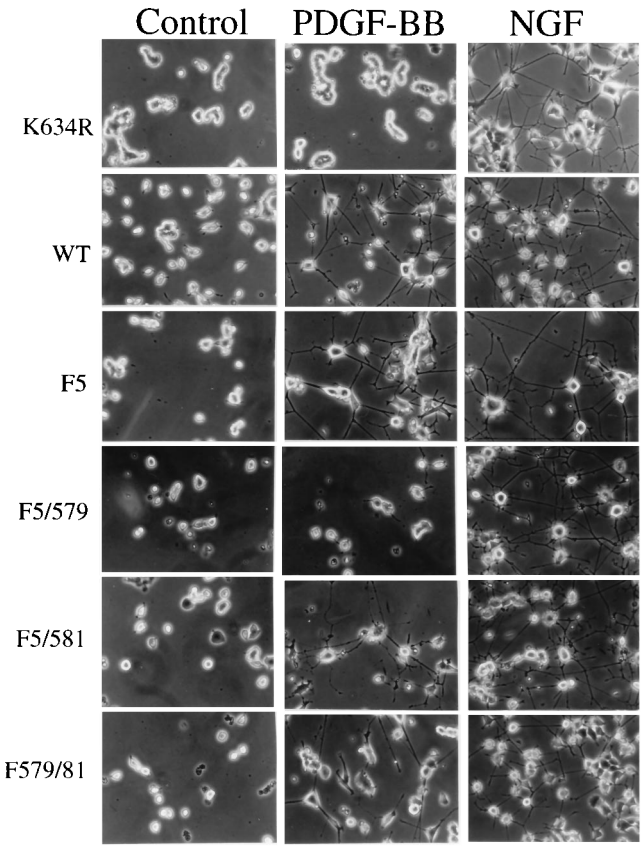

FIG. 2. PDGF-BB- and NGF-stimulated neurite outgrowth in PC12 cells expressing mutant βPDGF-Rs. PC12 clones expressing the indicated WT or mutant βPDGF-Rs were seeded on collagen-coated 35-mm dishes. Subsequently, the growth medium was replaced with Dulbecco's modified Eagle's medium containing 1% horse serum and either 30 ng of PDGF-BB per ml or 100 ng of NGF per ml as indicated. Fresh growth factor-containing medium was added after 3 days, and the cells were photographed after 6 days of treatment.

lowed multiple signaling pathways to be selectively abolished while other signals remained intact in response to activation of the mutant βPDGF-Rs. As a starting point for our receptor mutagenesis studies, five tyrosine-to-phenylalanine mutations were introduced in the βPDGF-R (referred to as F5 βPDGF-R). Mutations of tyrosines 740, 751, 771, 1009, and 1021 to phenylalanine in the F5 βPDGF-R have been well characterized in epithelial cells for their ability to associate with PI3-K, GAP, Syp, and PLCγ (68). Similarly, characterization of the F5 receptor in PC12 cells demonstrated that PI3-K, GAP, Syp, and PLCγ did not stably associate with this receptor when expressed in neuroendocrine cells in response to PDGF (data not shown). These results are summarized in Fig. 1.

Interestingly, in the absence of any binding of PI3-K, GAP, Syp, and PLCγ, the F5 βPDGF-R mediated differentiation of PC12 cells and the activation of MAP kinase (Fig. 2 and 4, respectively). In contrast, the F5 βPDGF-R activated MAP kinase in two different cell lines, canine kidney epithelial cells (TRMP) and human hepatoma cells (HepG2) (data not shown), but this receptor was unable to mediate a mitogenic response in these cells (68). This indicated that the signals from tyrosine kinase receptors which mediate cellular mitogenesis and differentiation are nonidentical, even though MAP kinase activation occurs in all three cell types. Our strategy was to use the F5 PDGF-R as a backbone for the introduction of other tyrosine-to-phenylalanine mutations in order to dissect additional pathways that are regulated by the PDGF-R and required for PC12 cell differentiation. Additional tyrosine-to-phenylalanine mutations were introduced into the βPDGF-R with positions of the corresponding phenylalanines illustrated in Fig. 1.

Neurite outgrowth assay. PC12 cells were analyzed for neurite outgrowth, and all of the PC12 cells expressing various mutant βPDGF-Rs respond to NGF stimulation of the endogenous Trk receptor with neurite outgrowth (Fig. 2). PC12 cells that express the K634R (Fig. 2) and F5/579/81 (data not shown) receptors differentiated in response to NGF but did not differentiate in response to PDGF. PDGF induced the extension of neurites and growth cones in cells that express the WT, F579/81, F5, and F5/581 βPDGF-Rs (Fig. 2). In contrast, the F5/579 receptor failed to mediate PDGF-dependent differentiation as characterized by neurite outgrowth. The inability to mediate differentiation was not due to the overall reduction in receptor kinase activity of the F5/579 receptor, as this receptor has more relative kinase activity than the F579/81 receptor (see below). Furthermore, PDGF-stimulated kinase activity is not a sufficient activity to mediate differentiation. Thus, the F5/579 PDGF-R is kinase active but has lost specific signal-transducing functions that are required for PC12 cell differentiation.

βPDGF-R expression. The WT βPDGF-R was introduced into PC12 cells as a means to characterize tyrosine kinase receptor-mediated PC12 cell differentiation since parental PC12 cells express neither αPDGF-R or βPDGF-R (20). Tyrosine-to-phenylalanine mutations were introduced into the βPDGF-R by site-directed mutagenesis. By using the retroviral expression vector pLXSN (48), PC12 cells were infected with

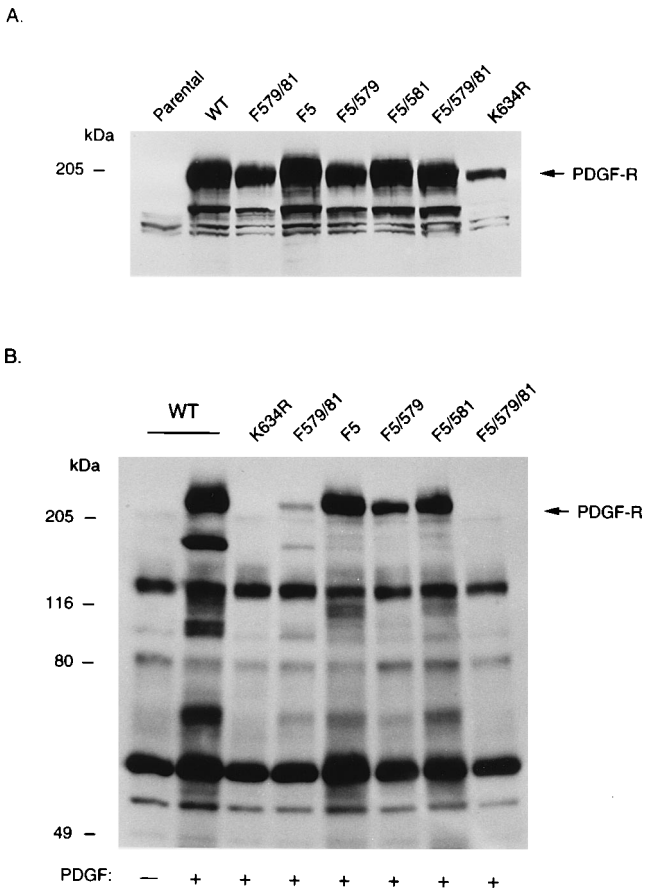

FIG. 3. Immunoblotting for expression of the various mutant PDGF-Rs in PC12 cells and tyrosine phosphorylation after stimulation with PDGF. (A) PC12 cell lysates (100 μg) were resolved by SDS–8% PAGE and transferred to nitrocellulose, and the proteins were probed with antibody directed against the carboxyl-terminus of the βPDGF-R. (B) Quiescent PC12 cells were stimulated with PDGF for 10 min at 37°C. Following cell lysis, 100 μg of cellular protein was separated by SDS-PAGE and transferred to nitrocellulose, and then proteins were probed with antiphosphotyrosine antibody (Upstate Biotechnology, Inc.). Immunoreactive proteins were visualized by autoradiography after probing with ^{125}I-protein A.

retroviruses encoding various mutated βPDGF-Rs. After neomycin drug selection, clones were picked and propagated and then screened by immunoblotting for βPDGF-R expression. The nomenclature that is used to describe each mutant βPDGF-R is depicted in Fig. 1.

PC12 cell clones that expressed similar levels of different mutant βPDGF-R were used for biochemical assays of receptor signaling and differentiation. Figure 3A shows an immunoblot of cell lysates from parental PC12 cells compared with cells that express the WT βPDGF-R and different mutant receptors. As expected, the parental PC12 cells have no detectable PDGF-Rs (Fig. 3A). Cells that express the WT βPDGF-R and all the clones that express tyrosine-to-phenylalanine receptor mutations expressed similar levels of receptor protein. PC12 cells that express the K634R kinase-inactive receptor had somewhat lower levels of receptor. In all of the PC12 cell clones, the mature, glycosylated βPDGF-R has a molecular mass of 205 kDa (Fig. 3A).

Antiphosphotyrosine immunoblotting of the βPDGF-R. Antiphosphotyrosine immunoblotting was performed on PC12 cell lysates to assess βPDGF-R kinase activity and tyrosine phosphorylation. Quiescent PC12 cells that express mutant PDGF-Rs were stimulated with PDGF for 10 min, and lysates were immunoblotted with antiphosphotyrosine antibody. WT βPDGF-R-expressing clones were also analyzed in the absence of PDGF stimulation to measure the basal levels of phosphotyrosine in the receptor and other proteins (Fig. 3B). The basal levels of phosphotyrosine in the mutant PDGF-Rs were similar to that shown for the quiescent WT receptor (data not shown). The 205-kDa tyrosine-phosphorylated βPDGF-R, which is indicated by an arrow in Fig. 3B, was detected by ^{125}I-protein A. Quantitation of phosphorylated PDGF-Rs was performed by using a PhosphorImager, and the data are expressed as relative units. PDGF stimulation of PC12 cells expressing the WT βPDGF-R had 13.7 relative units of phosphotyrosine content compared with unstimulated cells. The F5 βPDGF-R had 11.5 relative units of phosphotyrosine content, which suggests that mutation of five major autophosphorylation sites does not significantly alter the autophosphorylation activity at other tyrosines of the F5 βPDGF-R. Addition of a tyrosine-to-phenylalanine mutation in either the 579 or 581 juxtamembrane position of the F5 βPDGF-R decreased receptor autophosphorylation activity. The F5/579 and F5/581 PDGF-Rs were autophosphorylated 5.7 and 8.1 relative units over basal levels, respectively. Phosphorylation of these receptors in response to PDGF was approximately half that of the WT receptor. Thus, mutations in the juxtamembrane domain appear to alter receptor kinase and autophosphorylation activity. This finding is consistent with previous data that showed decreased kinase activity in the macrophage colony-stimulating factor receptor that had tyrosines mutated to alanine in the juxtamembrane domain where the βPDGF and the macrophage colony-stimulating factor receptors are highly homologous in this region (51).

The F579/81 βPDGF-R, which has two mutations in the juxtamembrane domain, had low, but detectable, levels of receptor tyrosine phosphorylation. The kinase activity of the F579/81 receptor in PC12 cells was easily detectable with antiphosphotyrosine antibodies. The amount of phosphotyrosine in the F579/81 receptor was 2.4 relative units, which is 5.5-fold less than that of the WT receptor, which presumably reflects an impaired autophosphorylation activity of this receptor. In contrast, Mori et al. reported that the F579/81 βPDGF-R, when expressed in porcine aortic endothelial cells, had no detectable kinase activity as assessed by in vitro kinase assay (50). Importantly, the F579/81 βPDGF-R retained biological activity by mediating neurite outgrowth in response to PDGF despite its diminished tyrosine phosphorylation (Fig. 2).

Predictably, cells with the kinase-inactive receptor, K634R, showed no tyrosine phosphorylation of the receptor, but surprisingly, cells with the F5/579/81 receptor also had no detectable receptor tyrosine phosphorylation. These data indicate that the F5/579/81 PDGF-R cannot measurably autophosphorylate itself and is thus functionally inactive in PC12 cells. Similar results that are shown in Fig. 3B were obtained when the βPDGF-R was immunoprecipitated with receptor antibody and then immunoblotted with antiphosphotyrosine antibody (data not shown).

As a second measure of the kinase activity of the various mutant βPDGF-Rs, a glutathione *S*-transferase (GST)–PLCγ fusion protein was used as an exogenous substrate. The various mutant βPDGF-Rs were immunoprecipitated with receptor antibody from quiescent and PDGF-stimulated lysates, and GST-PLCγ was phosphorylated in the presence of [γ-^{32}P]ATP (data not shown). Quantitation was performed by using a PhosphorImager. The phosphorylation of GST-PLCγ relative to the respective basal receptor activity was as follows: WT, 10; F579/81, 2.1; F5, 6.1; F5/579, 4.6; and F5/581, 5.2. The phos-

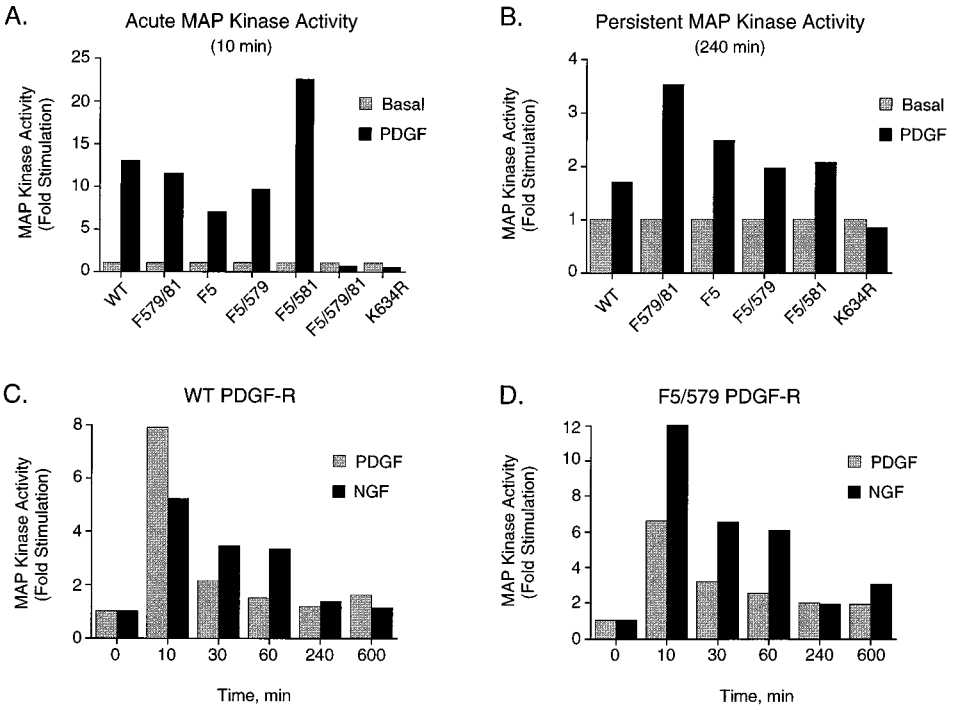

FIG. 4. Acute and persistent activation of MAP kinase. PC12 cells expressing the various mutant PDGF-Rs were stimulated with PDGF at 37°C for 10 min (A), 4 h (B), or the indicated times (C and D). Cell lysates were adsorbed to DEAE-Sephacel where p42 and p44 MAP kinase were eluted, and the substrate EGF-$R_{662-681}$ peptide was phosphorylated in the presence of [γ-^{32}P]ATP as described in Materials and Methods.

phorylation of GST-PLCγ by the F579/81 receptor was easily detectable and was the lowest of all the mutant receptors. The rank order for phosphorylation of an exogenous substrate and for receptor autophosphorylation, as shown in Fig. 3B, was similar for the various mutant receptors (WT and F5 > F5/579 and F5/581 > F579/81). Thus, these various mutant βPDGF-Rs are capable of incorporating phosphate into an exogenous substrate to the same relative extent as they incorporate phosphate into themselves by autophosphorylation. Significantly, the low kinase activity of the F579/81 βPDGF-R was still sufficient for this receptor to mediate neurite outgrowth. The conclusion from this analysis is that modest kinase activity and tyrosine phosphorylation of the PDGF-R is sufficient for mediating PC12 cell differentiation.

Transient and persistent activation of MAP kinase and tyrosine phosphorylation of PLCγ. As an initial screen for mutant PDGF-R activity, we analyzed PDGF-dependent MAP kinase activity in PC12 cells expressing the various mutant βPDGF-Rs. Stimulations were performed at 10 min since this was a time point at which all receptors maximally activated MAP kinase (data not shown). Cell lysates were prepared and applied to DEAE-Sephacel columns in which p42 and p44 MAP kinases were eluted and assayed (Fig. 4A). All of the mutant βPDGF-Rs, except the kinase-inactive F5/579/81 and K634R receptors, mediated the activation of MAP kinase. Even the F5/579 βPDGF-R, which does not mediate PC12 cell differentiation, activates MAP kinase 10-fold. There were different levels of MAP kinase activation between the various mutant βPDGF-Rs, and Fig. 4A is representative of the results from three independent experiments. The extent of MAP kinase activation by PDGF was similar to that obtained for EGF receptor stimulation (data not shown).

Persistent activation of MAP kinase has been predicted to be critical for signaling PC12 cell differentiation (20, 63). Cells were stimulated with PDGF for 4 h to determine whether the mutant PDGF-Rs could mediate persistent activation of MAP kinase (Fig. 4B). Consistent with the transient MAP kinase results, all of the mutant PDGF-Rs that have tyrosine kinase activity persistently activate MAP kinase. Significantly, the F5/579 PDGF-R was capable of mediating persistent MAP kinase activity similar to that of WT receptors even though this receptor does not mediate PC12 cell differentiation. Furthermore, the WT and F5/579 βPDGF-Rs mediate a persistent activation of MAP kinase similar to that mediated by the NGF receptor after 10 h of growth factor treatment (Fig. 4C and D). These data indicate that persistent activation of MAP kinase activity in response to receptor stimulation, in contrast to robust overexpression of activated MEK, is not sufficient to induce PC12 cell differentiation.

Tyrosine phosphorylation of PLCγ correlates with stimulation of phosphatidylinositide hydrolysis (36, 69), and PLCγ activity has been shown to be persistently stimulated in PC12 cells in response to NGF (58), indicating that this event is correlated with the differentiation response. For this reason, we examined the kinetics of tyrosine phosphorylation of PLCγ in PC12 cells expressing the various mutant βPDGF-Rs. Stimulation of the WT βPDGF-R resulted in tyrosine phosphorylation of PLCγ (Fig. 5A). In contrast, stimulation of the F579/581 βPDGF-R for 10 min resulted in diminished, but significant, tyrosine phosphorylation of PLCγ relative to that with the WT receptor, which may reflect the diminished kinase activity of this receptor (Fig. 5A). Stimulation of the F5, F5/579, and F5/581 PDGF-Rs, which have tyrosine 1021 mutated to phenylalanine, did not induce tyrosine phosphorylation of PLCγ, most probably because the receptors do not associate with PLCγ (Fig. 5A). PLCγ is persistently tyrosine phosphorylated in response to stimulation of the WT and F579/81 βPDGF-Rs (Fig. 5B) where both of these receptors mediate

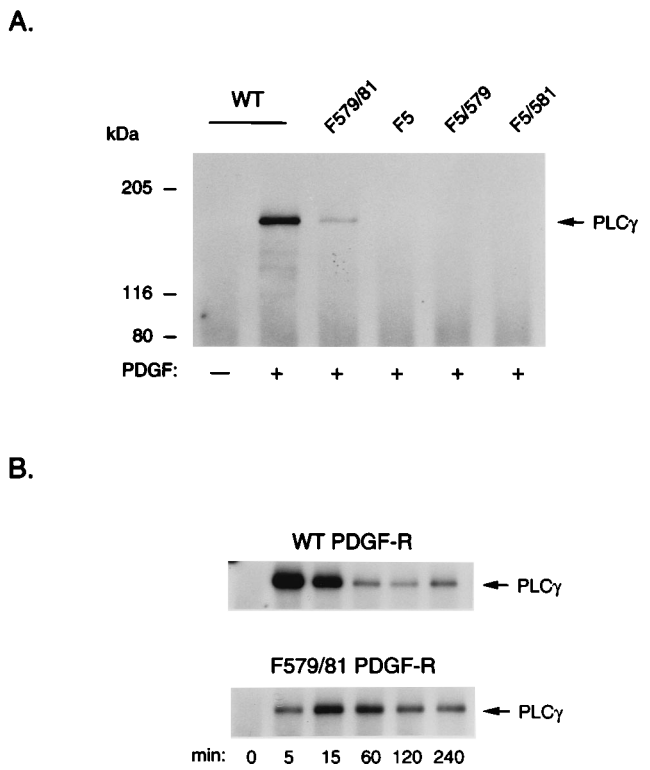

FIG. 5. (A) Tyrosine phosphorylation of PLCγ. Antiphosphotyrosine immunoprecipitation and immunoblotting of PLCγ demonstrate the activation of PLCγ that is mediated by mutant βPDGF-Rs. (B) Time course of PLCγ tyrosine phosphorylation. PC12 cells expressing the WT and the F579/81 βPDGF-R were assayed for the persistent tyrosine phosphorylation of PLCγ.

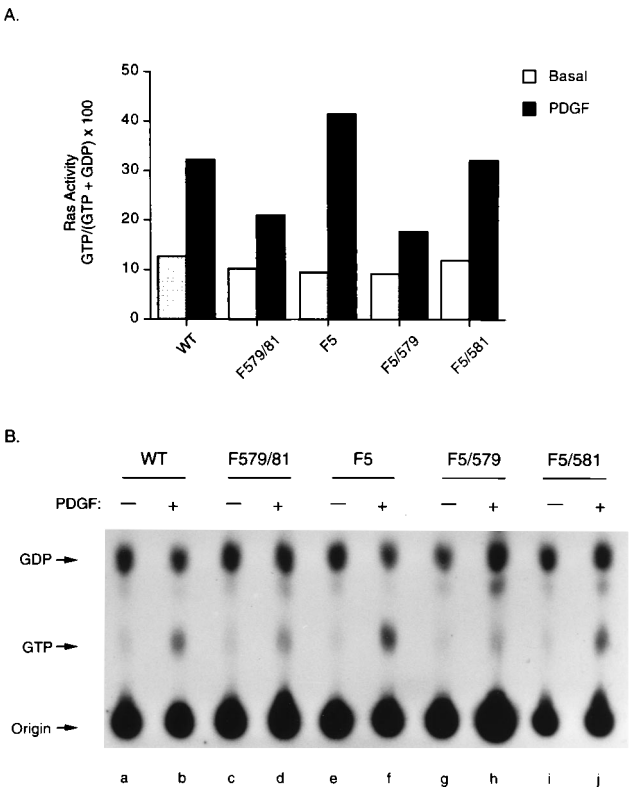

FIG. 6. GTP loading onto p21^{ras} after PDGF stimulation. PC12 cells expressing the various mutant PDGF-Rs were radiolabeled with $^{32}PO_4$ and stimulated with PDGF for 10 min. p21^{ras} was immunoprecipitated with monoclonal antibody Y13-259, and guanine nucleotides were resolved by polyethyleneimine-cellulose thin-layer chromatography with 0.75 M KH_2PO_4 as solvent. GDP and GTP were resolved adjacent to the immunoprecipitates in order to identify the radioactive nucleotides. PhosphorImager quantitation and the autoradiogram are shown in panels A and B, respectively.

neurite outgrowth. In contrast, the F5/579 receptor does not activate PLCγ and cannot mediate neurite outgrowth, even though it mediates persistent MAP kinase activation. These results indicate that the differentiation response requires persistent activation of the MAP kinase pathway and a second signal response that may correlate with PLCγ activity.

Ras activation. The ability of the various mutant βPDGF-Rs to activate Ras was determined by radiolabeling PC12 cells with $^{32}PO_4$ and immunoprecipitating p21^{ras} to measure the amount of GTP bound to activated p21^{ras}. Figure 6 shows that the mutant βPDGF-Rs activated the GTP loading of Ras by two- to fourfold relative to basal levels of Ras · GTP. The F5/579/581 and K634R (data not shown) receptors are inactive and did not stimulate GTP binding to Ras. While GTP loading onto Ras was readily detected with the F5/579 βPDGF-R, the PDGF-stimulated level of active Ras was only 50% of the WT level. Since the F5/579 receptor was unable to mediate PC12 cell differentiation, these results suggested that a certain level of Ras activation may be necessary, but is not sufficient, for growth factor receptor-mediated PC12 cell differentiation. To investigate this possibility, we examined a number of Ras-dependent events in the PC12 cells expressing the βPDGF-Rs.

B-Raf and Raf-1 activation. PC12 cells were challenged with PDGF to determine if any of the mutations in the βPDGF-R would affect activation of B-Raf and Raf-1. For B-Raf assays, PC12 cells were stimulated with PDGF for 10 min at 37°C and immunoprecipitated B-Raf was used to phosphorylate kinase-inactive MEK-1 (Fig. 7A). The WT, F5, and F5/581 βPDGF-Rs mediated 4.6-, 3-, and 2.8-fold activation of B-Raf, respectively (Fig. 7A, lanes b, f, and j). These results are expressed as PhosphorImager units relative to the respective basal levels of B-Raf activity (Fig. 7B). In contrast, the F579/81 and F5/579 receptors have reproducibly mediated a modest 1.4- and 1.9-fold activation of B-Raf (lanes d and h). In similar experiments, immunoprecipitated Raf-1 was used to phosphorylate recombinant kinase-inactive MEK-1 (Fig. 8). The results obtained with Raf-1 correspond with those obtained with B-Raf. The WT, F5, and F5/581 βPDGF-Rs mediate a 2.9-, 2.1-, and 3.3-fold activation of Raf-1, respectively (Fig. 8, lanes b, f, and j), whereas the F579/81 and F5/579 receptors activate Raf-1 by 1.3- and 1.5-fold, respectively (lanes d and h). The low level of B-Raf and Raf-1 activation in cells with the F579/81 and F5/579 PDGF-Rs correlates with mutation of Tyr-579.

We have previously shown that the activation of MAP kinase can be dissociated from the activation of B-Raf and Raf-1 in PC12 cells (65). The F579/81 and F5/579 PDGF-Rs mediate the activation of MAP kinase to a similar extent as the wild-type βPDGF-R, and yet these receptors consistently activated B-Raf and Raf-1 to a lesser extent than the WT receptor. It must be remembered that the F5/579 βPDGF-R does not mediate PC12 cell differentiation, indicating that the diminished, yet detectable, Ras, B-Raf, and Raf-1 activation is not the basis for loss of neurite outgrowth observed with this receptor.

Src association with the βPDGF-R and tyrosine phosphorylation. To investigate the ability of the various mutant PDGF-Rs to associate with p60$^{c\text{-}src}$, we performed the follow-

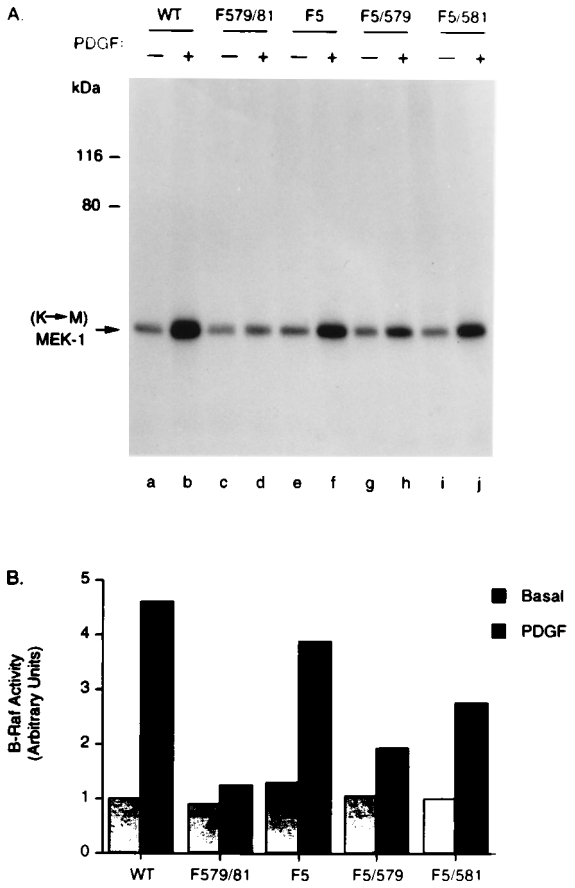

FIG. 7. B-Raf phosphorylation of kinase-inactive MEK-1. PC12 cells expressing the various mutant PDGF-Rs were stimulated with PDGF for 10 min at 37°C. B-Raf was immunoprecipitated and incubated with recombinant kinase-inactive MEK-1 in the presence of [γ-^{32}P]ATP. The in vitro kinase reactions were resolved by SDS–10% PAGE; the autoradiogram is shown in panel A, and the PhosphorImager quantitation is shown in panel B.

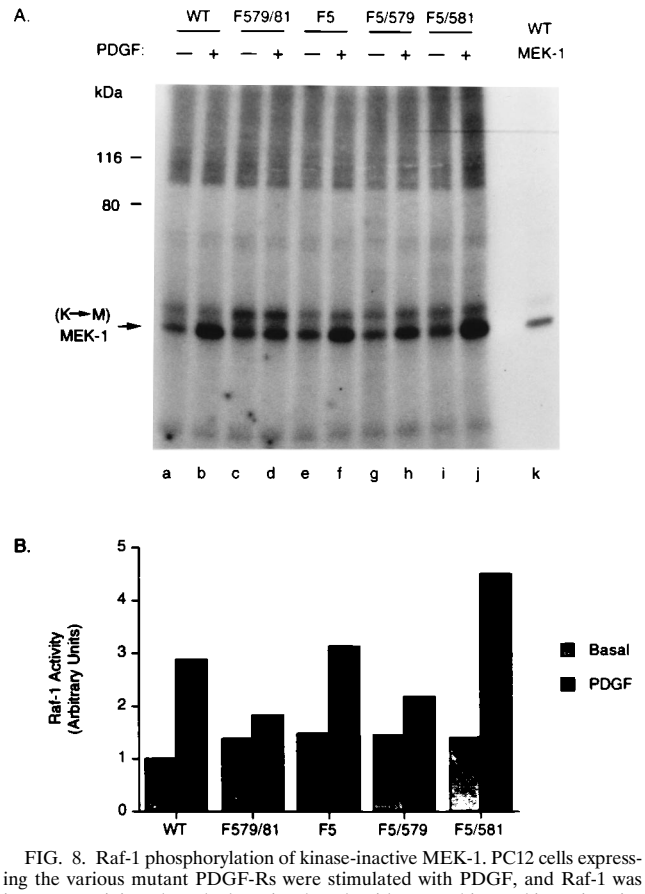

FIG. 8. Raf-1 phosphorylation of kinase-inactive MEK-1. PC12 cells expressing the various mutant PDGF-Rs were stimulated with PDGF, and Raf-1 was immunoprecipitated and then incubated with recombinant kinase-inactive MEK-1 in the presence of [γ-^{32}P]ATP. The in vitro kinase reactions were resolved by SDS–10% PAGE; the autoradiogram is shown in panel A, and the PhosphorImager quantitation is shown in panel B. Recombinant WT MEK-1 was phosphorylated with [γ-^{32}P]ATP and used as a marker in lane k to indicate the position of kinase-inactive MEK-1.

ing experiments. PC12 cells that express the WT βPDGF-R were stimulated with PDGF, the receptor was immunoprecipitated, an in vitro kinase assay was performed, and then the samples were reimmunoprecipitated with p60$^{c\text{-}src}$ antibody. The more traditional approach of immunoblotting PDGF-R immunoprecipitates with p60$^{c\text{-}src}$ antibodies was problematic and did not give consistent results. Stimulation with PDGF leads to association of p60$^{c\text{-}src}$ with the βPDGF-R and tyrosine phosphorylation of p60$^{c\text{-}src}$ (Fig. 9). The specificity of the assay was confirmed when an immunoprecipitation from PDGF-stimulated cells, as shown in lane c of Fig. 9, was performed in the absence of p60$^{c\text{-}src}$ monoclonal antibody 327 where no p60$^{c\text{-}src}$ phosphorylation is observed.

The activated WT and F5 receptors associated with p60$^{c\text{-}src}$ and stimulated p60$^{c\text{-}src}$ tyrosine phosphorylation (Fig. 9, lanes b and g). This result demonstrates that tyrosines 740, 751, 771, 1009, and 1021 are not critical for mediating βPDGF-R association with p60$^{c\text{-}src}$. Mutation of tyrosine 579 in the background of the F5 βPDGF-R eliminated detectable p60$^{c\text{-}src}$ association with the receptor (Fig. 9, lane i). In contrast, mutation of tyrosine 581 in the F5 βPDGF-R only partially reduced p60$^{c\text{-}src}$ association with the βPDGF-R. The F5/579 and F5/581 receptors have similar in vivo autophosphorylation activities as demonstrated in Fig. 3. Biologically, the F5/579 and F5/581 receptors differ in that the F5/579 receptor does not mediate neurite outgrowth. Thus, the ability of p60$^{c\text{-}src}$ to associate with the βPDGF-R in the F5 background demonstrates a critical role for p60$^{c\text{-}src}$ in receptor-mediated PC12 cell differentiation.

Curiously, elimination of just the p60$^{c\text{-}src}$ component of the βPDGF-R signaling cascade is not sufficient to disrupt receptor-mediated neurite outgrowth. The association of p60$^{c\text{-}src}$ with the F579/81 receptor is not detectable, and this receptor still mediates PC12 cell differentiation despite a low intrinsic receptor kinase activity (Fig. 9, lane e). Note that the F579/81 receptor is able to activate PLCγ (Fig. 5), suggesting that PLCγ activation can complement the Src pathway, which is not detectable in the F579/81 βPDGF-R, and therefore allows this receptor to mediate differentiation. Furthermore, the extent of intrinsic receptor kinase activity is not a limiting step in receptor-mediated PC12 cell differentiation; rather, specific autophosphorylation sites and activity towards specific substrates are the critical determinants.

DISCUSSION

Our results demonstrate that the Ras/Raf/MAP kinase pathway is not sufficient for PC12 cell differentiation. Growth factor tyrosine kinase receptor stimulation of the Ras/Raf/MAP kinase pathway is not sufficient to mediate PC12 cell differenti-

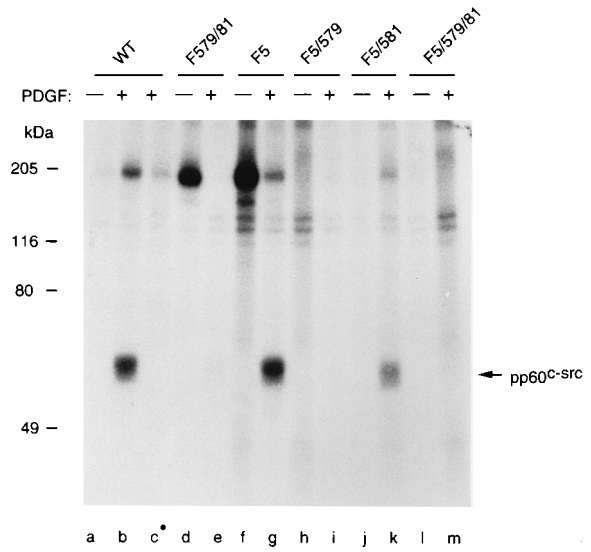

FIG. 9. Association and phosphorylation of p60$^{c\text{-}src}$ with the PDGF-R. PC12 cells expressing the various mutant PDGF-Rs were stimulated with PDGF for 10 min at 37°C. The βPDGF-R was immunoprecipitated from cell lysates, and an in vitro kinase assay was performed with [γ-^{32}P]ATP. The immune complexes were disrupted by heating at 100°C for 3 min in 0.5% SDS–1 mM dithiothreitol. Insoluble protein A was removed by centrifugation, and p60$^{c\text{-}src}$ was immunoprecipitated with monoclonal antibody 327. Immunoprecipitated proteins were resolved by SDS–10% PAGE, and the gel was treated with alkali as described in Materials and Methods. The immunoprecipitates in lanes b and c were treated the same except that monoclonal antibody 327 was omitted in lane c (•).

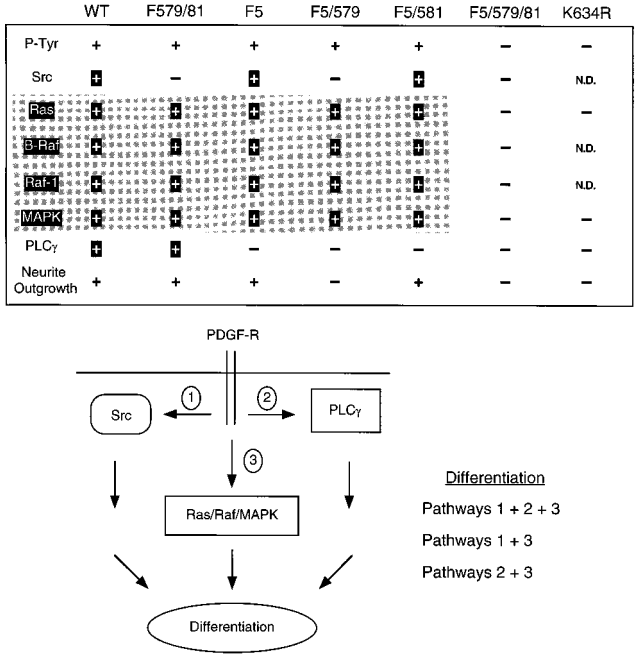

FIG. 10. Summary and model of the differentiation and signal transduction pathways mediated by mutant PDGF-Rs. The βPDGF-R activates a number of signal transduction pathways in PC12 cells which ultimately lead to neurite outgrowth. Mutant PDGF-Rs demonstrate the requirement for the Ras/Raf/MAP kinase pathway (numeral 3 in figure) (shaded area, above). In the absence of the Src pathway (numeral 1 in figure), the PLCγ pathway (numeral 2 in figure) can rescue PC12 cell differentiation (black box under the F579/81 receptor). Conversely, in the absence of the PLCγ pathway (numeral 2 in figure), Src can rescue PC12 cell differentiation (black boxes under the F5 or F5/581 receptor). The K634R and F5/579/81 mutant PDGF-Rs also demonstrate a requirement for receptor tyrosine kinase activity for PC12 cell differentiation. For an explanation of the plus and minus signs and "N.D.," please see the legend to Fig. 1.

ation as measured by neurite outgrowth and Na^+ channel activity (14). Functional Na^+ channel expression correlates with, but is distinct from, morphological differentiation of PC12 cells (13). The findings indicate that activation of the Ras/Raf/MAP kinase pathway and additional signaling events are required for morphological differentiation.

Previous work had demonstrated that functional Ras activation was required for PC12 cell differentiation (61, 72). Furthermore, oncogenic and constitutively active mutants of Ras, Src, Raf, and MEK were capable of differentiating PC12 cells (3, 10, 62, 71), and microinjection of Src antibodies inhibited PC12 cell differentiation in response to NGF (38). Our results indicate that Src activation by the PDGF-R is not required for PC12 cell differentiation but do not exclude the possibility that Src functions in other aspects of the differentiation process which has been suggested for neurite outgrowth of *src*-negative cerebellar neurons (26). These functions would require Src regulation independent of receptor stimulation.

The ability of oncogenic forms of Ras and Src to induce PC12 cell differentiation is undoubtedly related to the high-level expression and constitutive activity of these proteins. It is clear that high-level expression of activated Ras and Src dramatically alters the regulation of multiple effector pathways that do not reflect the temporal and quantitative responses observed with growth factor stimulation of receptor tyrosine kinases (53, 62). Similarly, high-level expression of activated MEK regulates the activity of MAP kinase pathways differently than that observed with growth factor receptor stimulation of this pathway (10).

The detailed analysis of signal transduction pathways required for PDGF-R-mediated PC12 cell differentiation indicates a requirement for multiple signals. Cumulatively, our results as summarized in Fig. 10, and those of others, indicate that stimulation of the Ras/Raf/MAP kinase pathway and Src and/or PLCγ activities are involved in PC12 cell differentiation (54, 60). This conclusion is founded on the findings that (i) expression of dominant inhibitory N^{17}Ras prevents growth factor-mediated activation of MAP kinase and neurite extension (61, 72), (ii) Syp regulation and GAP (43) are not required for neurite extension, (iii) PI3-K activity is not required for neurite extension (37), (iv) PLCγ and MAP kinase are the two known effector systems persistently stimulated by both NGF receptors and PDGF-Rs, and (v) when p60$^{c\text{-}src}$ interaction with the PDGF-R is lost, then PLCγ stimulation is required to observe neurite outgrowth.

The requirement of Src or PLCγ activation is not simply related to maintaining a persistent MAP kinase activity. The F5/579 mutant PDGF-R has a persistent MAP kinase activity similar to that observed with the WT receptor, but this receptor does not differentiate in response to PDGF. The significant feature of the F5/579 βPDGF-R is that it does not associate with either Src or PLCγ. Therefore, this receptor demonstrates a requirement for persistent MAP kinase activity and the Src or PLCγ pathways for PC12 cell differentiation. It is possible that there is an undefined signaling pathway, whose regulation overlaps with Src and PLCγ, that is involved in the differentiation pathway. Nevertheless, the Src and PLCγ pathways appear to be interchangeable to rescue PC12 cell differentiation as demonstrated by the F5, F579/81, and F5/579 βPDGF-Rs (Fig. 10). Interestingly, PLCγ, in addition to MAP kinase, is persistently activated by growth factors that differentiate PC12 cells (58), although much more attention has been recently

given to persistent activation of the MAP kinase pathway. The targets of PLCγ regulation involved in PC12 cell differentiation are not defined but predictably involve protein kinase C enzymes. To date, the targets of Src which are independent of the Ras/Raf/MAP kinase pathway and involved in PC12 cell differentiation are also undefined.

The requirement of multiple signal transduction pathways, rather than high levels of intrinsic receptor kinase activity, provides the necessary integration of specific and distinct inputs to commit a cell to differentiation (Fig. 10). The Trk receptor, which is expressed at only 5,000 receptors per cell, has a sufficient and specific tyrosine kinase activity to recruit the necessary signal pathways for differentiation (24). Similarly, the F579/81 βPDGF-R demonstrates that low intrinsic receptor tyrosine kinase activity, which is sufficient to regulate specific signal pathways, is also sufficient to mediate neurite outgrowth.

Expression of dominant-negative N^{17}Ras has defined Ras as a necessary signaling component for the differentiation response (61, 72). Tyr-716 of the βPDGF-R is an autophosphorylation site which mediates the association of the receptor with Grb2 and Ras activation (1). In PC12 cells, the F5 βPDGF-R binds Grb2 correlating with Ras activation (data not shown). The F5/579 βPDGF-R activates Ras and MAP kinase, and yet the activation of these pathways is not sufficient to mediate PC12 cell differentiation. This result clearly indicates that additional signals are required for receptor-mediated PC12 cell differentiation.

Other growth factor receptor tyrosine kinases (i.e., EGF and insulin receptors) may regulate overlapping signaling pathways, but unlike oncogenic proteins such as activated Ras and MEK, temporal and quantitative differences in these signals allow selectivity in committing cells to specific phenotypic consequences. Thus, even though the EGF and insulin receptors can regulate the Ras/Raf/MAP kinase pathway and PLCγ activity, extremely high receptor levels are required for EGF and insulin receptor-induced PC12 cell differentiation (11, 64). This appears to be related to the temporal signaling properties and efficient down-regulation of the EGF receptor, for example, relative to the PDGF-R or NGF receptor (9, 20, 40). The EGF and insulin receptor responses, at modest receptor numbers, are insufficient to induce PC12 cell differentiation while modest EGF receptor expression can induce mitogenesis of fibroblasts and PC12 cells (25). These differential EGF responses occur at receptor numbers similar to those that are sufficient for the PDGF-R to stimulate PC12 cell differentiation or fibroblast growth.

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