Rat PC12 cells respond to extracellular peptide growth factors in at least two distinct ways. When treated with nerve growth factor (NGF) PC12 cells exit the cell cycle and differentiate to a neuronal phenotype, whereas when treated with epidermal growth factor, they proliferate. We examined the potential role of Src homology 2 (SH2)-containing protein tyrosine phosphatases (PTPs) in the differentiation process. PC12 cells express substantial amounts of both SH-PTP1 and 2. SH-PTP1, but not SH-PTP2, becomes tyrosine phosphorylated following NGF, but not epidermal growth factor treatment. The enzymatic activity of SH-PTP1 toward an exogenous substrate following NGF treatment is increased 2-fold. We found that SH-PTP1 binds to the NGF receptor TrkA in vitro and that anti-TrkA immunoprecipitates have PTP activity. These results show that SH-PTP1 is differentially phosphorylated and activated by NGF in PC12 cells and suggest that this activation may play a role in NGF-induced differentiation.

Diverse extracellular growth factors use one of a few common strategies to transmit their messages to the cell interior. Among the best understood of these strategies is that utilized by ligands that bind to receptor protein tyrosine kinases (RPTKs). The general scheme by which these signals are transduced includes receptor dimerization and autophosphorylation, followed by the recruitment of an array of Src homology 2 (SH2)-containing molecules (1). These recruited SH2-containing proteins include enzymes such as kinases, phosphatases, GTPase-activating proteins, and phospholipases, and adaptor proteins, which bind to activators of the Ras family of GTPases, thus linking the signaling complex to downstream effectors (2).

One of the SH2-containing proteins recruited by several different RPTKs is protein tyrosine phosphatase (PTP) SH-PTP2 (3-6). In the case of the platelet-derived growth factor (PDGF)-β receptor SH-PTP2 binds to phosphorylated tyrosine 1009, and itself becomes tyrosine phosphorylated by the receptor (7, 8). Tyrosine-phosphorylated SH-PTP2 then serves as a docking site for the SH2-containing adaptor protein Grb2 (8, 9). In this way, one function of SH-PTP2 binding to the PDGF receptor appears to be as a platform for the assembly of additional adaptor proteins required to generate an active signaling complex. The role of the phosphatase activity itself is not well established at present.

Although structurally similar to SH-PTP2, the second known SH2-containing PTP (SH-PTP1) (10-12) appears to have a different role in signal transduction. Disruption of SH-PTP1 function gives rise to the motheaten and viable motheaten mouse strains, which have severe immunologic defects as well as multiple other hematopoietic abnormalities (13-15). These mice exhibit a large increase in the number of myeloid precursor cells as a result of colony-stimulating factor-1-independent proliferation of macrophages and increased sensitivity of colony-forming units to erythropoietin. In addition, there is also an increase in the numbers of erythroid precursors, consistent with a defect in interleukin-3 signaling. Biochemical evidence shows that SH-PTP1 is coupled to c-Kit, interleukin-3, and the insulin receptors but not to the colony-stimulating factor-1 receptor (16-19). SH-PTP1 dephosphorylates a variety of RPTKs when coexpressed in 293 cells (5) and has been shown to down-regulate interleukin-3-induced tyrosine phosphorylation and mitogenesis (16). Thus, unlike SH-PTP2, this enzyme appears to act as a negative regulator of signal transduction.

In PC12 cells, engagement of the nerve growth factor (NGF) receptor TrkA results in differentiation of the cells to a neuronal phenotype, whereas engagement of the epidermal growth factor (EGF) receptor causes proliferation (20, 21). One possible reason for these differences could be that these receptors recruit and/or activate distinct populations of SH2 proteins. The role of SH2-containing PTPs in PC12 cell differentiation and proliferation has not previously been reported. We therefore examined the effect of NGF and EGF treatment on the receptor binding, phosphorylation, and activity of both known SH-PTPs in PC12 cells. We report here that both SH-PTP1 and 2 are present in these cells and that the NGF receptor tyrosine phosphatase SH-PTP2 phosphorylates and activates only SH-PTP1.

MATERIALS AND METHODS

Cell Culture—PC12 cells (obtained from S. Halegoua, Stony Brook, NY) were grown at 37 °C in a 5% CO₂ atmosphere in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated horse serum, 5% fetal bovine serum, and antibiotics. TrkA-overexpressing PC12 cells (6-24) were cultured as described (22). Rat1 fibroblasts were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, and rat lymphocytes (derived from a Moloney murine leukemia virus-induced rat thymoma) were maintained in...
RPMI supplemented with 10% fetal bovine serum. NGF and EGF were obtained from Harlan Bioproducts.

Northern Blots—Total RNA was prepared from cells using guanidine chloride and acid phenol (23). RNA (10 μg) was electrophoresed on a 1.0% agarose, 6% formaldehyde gel, transferred to a nylon membrane, and cross-linked using UV irradiation. Filters were hybridized overnight in 50% formamide, 7% SDS, 1% bovine serum albumin with [32P]cDNA-labeled human SH-PTP1 or 2 cDNA and washed twice at 0.2× SSC, 0.2% SDS at 55°C. Autoradiograms were obtained by exposing the blots to Kodak XAR film with intensifying screens at 70°C for 24 h.

Immunoprecipitations and Immunoblots—Cells were washed twice with cold phosphate-buffered saline and lysed by rocking for 10 min at 4°C with 0.6 ml/10-cm plate of Nonidet P-40 lysis buffer (50 mM Tris-HCl, pH 8.0, 137 mM NaCl, 1% Nonidet P-40, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin). Lysates were centrifuged at 13,000 g for 15 min at 4°C in a microcentrifuge. Protein concentration was determined using a commercial assay based on bicinchoninic acid (Pierce). Immunoprecipitations (300–600 μg of protein in 300–600-μl volume) were carried out at 4°C for a minimum of 1.5 h with gentle rocking. The immune complexes were collected on protein A-Sepharose (100 μl of a 10% solution) for 45 min. The beads were washed three times with lysis buffer and one time with water, Nonidet P-40 lysis buffer, or PTP assay buffer.

Proteins were separated in 8% SDS-polyacrylamide gels (SDS-PAGE). After separation, the proteins were transferred to a nitrocellulose membrane that was then stained with Coomassie Blue and lose membrane that was then stained with Coomassie Blue and lose membrane for the presence and relative levels of this enzyme. Northern blots were hybridized with an SH-PTP1 probe. B, 20 μg of cell lysate from Rat1 fibroblasts, PC12 cells, and rat lymphocytes were separated on an 8% SDS-PAGE gel. After transfer to a nitrocellulose membrane, an immunoblot was performed with either polyclonal anti-SH-PTP1 or monoclonal anti-SH-PTP2 antibodies.

that two of the three rat tissues tested contain a transcript of about the expected size. In agreement with previous reports, this transcript is most abundant in rat lymphocytes and nearly absent in fibroblasts (Fig. 1A). Densometric analysis of the Northern blot revealed that the amount of SH-PTP1 transcript in PC12 cells is about 35% of the amount found in rat lymphoblasts (not shown). A similar relationship was observed for rat SH-PTP1 protein. Equivalent amounts of protein from Rat1 fibroblasts, PC12, and rat lymphoblasts were electrophoresed and immunoblotted with antibodies against SH-PTP1 and 2. Fig. 1B shows that there are substantial levels of SH-PTP1 protein in PC12 cells but not in Rat1 fibroblasts, whereas SH-PTP2 is present at similar levels in all three tissues. SH-PTP1, but Not SH-PTP2, Is Tyrosine Phosphorylated in a NGF-dependent Manner—As PC12 cells contain both known SH-PTPs, we sought to determine whether either of these enzymes couples to TrkA and/or becomes tyrosine phosphorylated in response to NGF. To maximize the sensitivity of the assay, we used 6-24 cells, which express about 10-fold more TrkA than the parental PC12 cell line, because of transfection with a TrkA expression vector (22). NGF induced an approximately 3-fold increase in the tyrosine phosphorylation of SH-PTP1, but not SH-PTP2, in 6-24 cells (Fig. 2B, right versus left lanes). Preincubation of SH-PTP1 with recombinant GST-SH-PTP1 protein prior to immunoprecipitation of the 6-24 lysate abolished the subsequent ~65-kDa band in both anti-SH-PTP1 and anti-phosphotyrosine blots (not shown). TrkA immunoprecipitates contain a tyrosine-phosphorylated protein with the same relative mobility as SH-PTP1 (Fig. 2A), left panel, second lane), but this may represent an isoform of Shc or other TrkA-associated protein (28–30), as no signal was seen in anti-SH-PTP1 blots of TrkA immunoprecipitates (Fig. 2A, middle panel). NGF, but not EGF, also stimulated tyrosine phosphorylation of SH-PTP1 in PC12 cells (Fig. 2C). SH-PTP1 phosphorylation was maximal at 25–50 ng/ml NGF (Fig. 3B). In addition to SH-PTP1, at least two other proteins that coprecipitate with SH-PTP1 become tyrosine phosphorylated on exposure of cells to NGF. The sizes of these proteins are about 110 and 140 kDa (Fig. 3A, sixth lane). Although the 110- and 140-kDa bands might represent TrkA and its precursor, TrkA antibody failed to recognize either of these bands. Tyrosine phosphorylation of SH-PTP1 and its associated proteins increased with increased length of exposure of cells to NGF (Fig. 3A). SH-PTP1 protein levels were unaffected by NGF treatment (bottom panels of A and B).

**FIG. 1. SH-PTP1 mRNA and protein are present in PC12 cells.** A, 10 μg of total RNA isolated from Rat1 fibroblasts, PC12 cells, and rat lymphocytes were separated on a denaturing agarose gel. The resulting Northern blot was hybridized as described under "Materials and Methods" with a 32P-labeled human SH-PTP1 probe. B, 20 μg of cell lysate from Rat1 fibroblasts, PC12 cells, and rat lymphocytes were separated on an 8% SDS-PAGE gel. After transfer to a nitrocellulose membrane, an immunoblot was performed with either polyclonal anti-SH-PTP1 or monoclonal anti-SH-PTP2 antibodies.

**RESULTS**

Both SH-PTP1 and 2 Are Present in PC12 Cells—Several growth factor receptors are known to couple directly to SH-PTP2, which is the predominant SH2-containing PTP in most tissues (3). Although the distribution of a second SH2-containing PTP (SH-PTP1) is thought to be restricted mainly to cells of hematopoietic lineage (12), we examined PC12 cells for the presence and relative levels of this enzyme. Northern blots containing RNA from Rat1 fibroblasts, PC12 cells, and rat lymphocytes were hybridized with an SH-PTP1 probe. Although the size and sequence of the rat SH-PTP1 transcript has not been reported previously, in mouse this transcript is 2.6 kilobases (12), and is 94% identical at the nucleotide level to the human mRNA within the coding region. Our results show
SH-PTP1 Is Activated by NGF Treatment—It has been reported that phosphorylation of SH-PTP1 leads to its activation (18). We tested the PTP activity of SH-PTP1 immunoprecipitated from control and NGF-treated 6-24 and PC12 cell lysates. The data summarized in Fig. 4 show that NGF causes a 2-fold increase in SH-PTP1 activity as compared with a very small (30%) increase in SH-PTP2 activity. In other (three) experiments and at a lower substrate concentration, NGF affected SH-PTP2 activity negatively, causing about a 10% decrease, whereas SH-PTP1 was still activated. Treatment of cells with EGF had no effect on SH-PTP1 activity (not shown). The small magnitude of SH-PTP1 activation by NGF is consistent with the results of Uchida et al. (18) and may be attributable at least in part to autodephosphorylation (26, 27).

TrkA Binds to SH-PTP1 in Vitro—SH-PTP1 protein was expressed as a GST-fusion protein in bacteria and purified by glutathione-agarose chromatography. This protein bound to TrkA from 6-24 cells in a NGF-dependent manner (Fig. 5). The GST-SH-PTP1 protein also bound activated TrkA from TrkA-infected Sf9 cell lysates (not shown), suggesting that in vitro the TrkA-SH-PTP1 interaction is not mediated through other cellular proteins. We could not, however, demonstrate the reverse of this reaction in vitro, binding of SH-PTP1 to TrkA immunoprecipitates from control and NGF-treated 6-24 cells. TrkA Immunoprecipitates from 6-24 Cells Contain a NGF-stimulated PTP Activity—Although we could not reliably detect SH-PTP1 protein in TrkA immunoprecipitates, we tested such precipitates for PTP activity. When assayed for PTP activity, TrkA immunoprecipitates from 6-24 cell lysates released 0.46 pmol of $^{32}$P from Raytide (Fig. 6). Treatment with NGF caused a 3-fold increase in activity. Preincubation of the TrkA antiserum with a peptide comprised of the carboxyl-terminal 14 amino acids of TrkA abolished detectable PTP activity in the subsequent immunoprecipitates (lane 3), as did the addition of 1 mM sodium vanadate to the TrkA immunoprecipitate (lane 4).
Regulation of SH-PTP1 in PC12 Cells

Although SH-PTP1 is usually thought of as a hematopoietic cell PTP (indeed, one of its many pseudonyms is HCP), its tissue distribution includes a variety of epithelial cell types in addition to cells of hematopoietic lineage (10–12). Here, we report that SH-PTP1 is also abundant in PC12 cells. Although we could not demonstrate binding of SH-PTP1 to TrkA in vitro, we show that this PTP is tyrosine phosphorylated and activated in NGF-treated PC12 cells and that it binds to TrkA in vitro in a NGF-dependent manner. It is interesting to note that Aparicio et al. (36) described the activation of three PTPs by NGF in PC12 cells, one of which has a molecular mass of about 60 kDa. Our results suggest that this PTP may be SH-PTP1.

Our findings are similar to those reported by Uchida et al. (18), who showed that SH-PTP1 is a direct substrate of the insulin receptor kinase. As in our studies with TrkA, in vitro binding of SH-PTP1 to the insulin receptor was shown to be ligand-dependent, and insulin stimulated the tyrosine phosphorylation and activity of SH-PTP1. These authors were not, however, able to detect direct binding of SH-PTP1 to the insulin receptor by coimmunoprecipitation. In our studies, it is possible that the Trk antibody partially displaced the Trk-bound PTP during immunoprecipitation. The Trk antibody used for these experiments was raised against the 14 carboxy-terminal amino acids of the TrkA receptor, where at least one of the phosphorylated tyrosines that is slated to transmit signals is located (28–30). Whether partially displaced by TrkA antibody or not, it is possible that the amount of SH-PTP1 bound to immunoprecipitated TrkA is too low to be detected by immunoblot using the polyclonal anti-SH-PTP1 antiserum. The activity assay used to detect the presence of PTPs in TrkA immunoprecipitates (Fig. 6) is much more sensitive than immunoblotting techniques and thus is not inconsistent with the coimmunoprecipitation results shown in Figs. 2A and 3A. A more detailed characterization of the PTP activity present in TrkA immunoprecipitates may resolve this issue.

As none of the known TrkA autophosphorylation sites matches the consensus for SH-PTP1 binding, the interaction between this PTP and TrkA may be mediated through an unconventional binding motif. Indeed, Uchida et al. (18) found that the binding of SH-PTP1 to the insulin receptor is mediated not through SH2-phosphotyrosine interactions but rather through an element in the carboxyl terminus of SH-PTP1. Alternatively, the NGF-induced tyrosine phosphorylation and activation of SH-PTP1 may be indirectly mediated by TrkA. In this scenario, NGF receptor activation leads to tyrosine phosphorylation of SH-PTP1 through an intermediate rather than by direct binding. For example, in PC12 cells the Src tyrosine kinase is activated by NGF and is required for differentiation (37). Matozaki et al. (38) have shown that Src can phosphorylate SH-PTP1 in vitro and that cells transformed with v-src have high levels of tyrosine-phosphorylated SH-PTP1, whereas nontransformed cells do not. Their results suggest that SH-PTP1 may be a direct target for the Src kinase. In murine T cells, similar findings have been noted regarding the Src-like protein kinase Lck and SH-PTP1 (27).

Although it has been suggested that the selection of differentiation versus proliferation pathways in PC12 cells is a result

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**Fig. 5.** NGF-dependent binding of TrkA to SH-PTP1 in vitro. GST-SH-PTP1 on glutathione-agarose beads was incubated with lysates (660 μg of protein) from control and NGF-treated 6-24 cells for 1 h at 4°C. The beads were washed five times with Nonidet P-40 lysis buffer and then boiled in SDS-PAGE sample buffer and chromatographed on an 8% SDS-PAGE gel. An immunoblot was performed with antibodies against TrkA.

**Fig. 6.** TrkA coimmunoprecipitates with NGF-stimulated PTP activity. Lysates from control (open rectangles) and NGF-treated (filled rectangles) 6-24 cells were immunoprecipitated with control serum (lane 1) or anti-TrkA (lanes 2–4). In lane 3, an excess of blocking peptide was added to the TrkA antibody prior to the immunoprecipitation; in lane 4, 1 μM sodium vanadate was added. The washed immunoprecipitates were assayed for PTP activity for 10 min using Raytide as substrate as described under "Materials and Methods." The data represent the average values obtained from two experiments.

From these data we infer that TrkA binds a PTP, although this experiment does not establish the identity of the bound PTP(s).

**DISCUSSION**

Upon exposure to NGF, PC12 (rat adrenal pheochromocytoma) cells differentiate into sympathetic nerve cells producing long neurites (20). We became interested in the possible role of PTPs in this signaling process. Like many other receptor PTKs, engagement of the TrkA by ligand leads to autophosphorylation of a number of specific residues in the carboxyl terminus of the receptor which serve as docking sites for SH2-containing proteins (28–30). There is ample precedent for involvement of SH-PTPs in signaling through many receptor PTKs. For example, SH-PTP2 binds to the activated PDGF-β receptor at tyrosine 1009 (7, 8). The bound PTP is phosphorylated by the receptor and serves as a docking site for the adaptor Grb2 (8, 9). SH-PTP2 appears to be required for PDGF signaling, as microinjection of neutralizing antisera or catalytically inactive forms of the enzyme inhibits PDGF-mediated mitogenesis in NIH-3T3 cells (31, 32). Similar results have been obtained regarding insulin-mediated signaling (33). The positive role of SH-PTP2 in generating growth signals has also been confirmed in developmental studies in both Drosophila and Xenopus (34, 35). In these organisms, disruption of SH-PTP2 function results in marked developmental abnormalities consistent with loss of function of RPTK-regulated pathways. Thus, SH-PTP2 appears to be a required element in some receptor PTK signaling pathways. Our findings in PC12 cells indicate that although SH-PTP2 is abundantly expressed, it does not associate with the NGF receptor, and it is neither tyrosine phosphorylated nor activated in response to NGF. Thus, it is unlikely that this PTP is involved in the regulation of differentiation in PC12 cells by NGF.
of quantitative differences among the PTK receptors (39), there must also be important qualitative differences, since native PC12 cells have approximately equal numbers of NGF and EGF receptors yet respond quite differently to each agent. Furthermore, certain proteins appear to be tyrosine phosphorylated in response to differentiation agents but not proliferative agents. To date, the most convincing example of such a protein is SNT, an 80-kDa p13 regulatory in response to differentiation agents but not proliferative agents. To date, the most convincing example of such a protein is SNT, an 80-kDa phosphotyrosine phosphatase (40), which is heavily tyrosine phosphorylated in response to NGF, but not EGF. To our knowledge, SH-PTP1 represents the second example of a protein of this type. Although we do not yet know if the tyrosine phosphorylation and activation of SH-PTP1 by NGF are required for PC12 differentiation, our results suggest that this enzyme plays a role in modulating NGF-induced signaling events.

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