Protein Tyrosine Phosphatase Activation during Nerve Growth Factor-induced Neuronal Differentiation of PC12 Cells

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Abstract

We have studied the role of protein tyrosine phosphatases (PTPases) during neuronal differentiation of PC12 cells. Nerve growth factor (NGF), a well-characterized differentiating agent for these cells, led to a decrease in DNA synthesis within 24 h. This was accompanied by a 2- to 3-fold increase in the activity of PTPases, measured as the dephosphorylation of polyacidic or polybasic substrates phosphorylated on tyrosine. PTPase activation was independent of cell density and proportional to NGF concentration, with a half-maximal effect occurring at 0.35 nM. High-performance liquid chromatography size exclusion chromatography revealed that PTPases with molecular masses of 550, 300, and 60 kilodaltons were activated in response to NGF. Additional studies showed that the presence of NGF made PC12 cells refractory to the mitogenic effect of epidermal growth factor. Our data indicate that NGF-induced neuronal differentiation and growth arrest in PC12 cells are associated with activation of several PTPases. We speculate that PTPase activation in response to NGF may inhibit the mitogenic actions of other growth factors.

Introduction

The PC12 clonal cell line, established by Greene and Tischler (1), was derived from a transplantable rat adrenal pheochromocytoma induced by X-ray irradiation (2). When grown in a medium containing nanomolar concentrations of NGF, PC12 cells stop dividing and reversibly differentiate into sympathetic nerve cells. This differentiation is characterized by extension of long, branching, neuronal-like varicose processes (1), electrical excitability (3), and increased neurotransmitter synthesis (4). Because of their response to NGF and their ability to survive in both differentiated and undifferentiated states, PC12 cells have become a widely used model for studying neuronal differentiation and the mechanism of action of NGF (for a review, see Ref. 5).

Enzyme-catalyzed reversible protein phosphorylation on tyrosine is an important mechanism for the regulation of cell growth, differentiation, and metabolism (6–8). Tyrosine kinase activity has been demonstrated for a number of growth factor receptors (7, 8), including the high-affinity NGF receptor (9). Reversal of tyrosine phosphorylation is catalyzed by PTPases. The net effect of protein phosphorylation on tyrosine is a balance between tyrosine kinases and PTPases (10). A regulatory role for PTPases in control of cell growth and differentiation has been implicated by in vitro studies on several cell lines (see "Discussion"). We hypothesized that NGF-induced differentiation of PC12 cells would be accompanied by an increase in PTPase activity, thus providing a potential link between NGF action and cessation of growth.

Results

Influence of Culture Conditions on PC12 Proliferation.

To interpret changes in PTPase activity during cell proliferation and differentiation, and since PC12 cells may vary between laboratories (11), we obtained data on the effects of culture conditions, cell density, and NGF on rate of PC12 cell growth and cell morphology. PC12 cells exhibit behavior consistent with autocrine growth factor production. This includes optimization of growth by conditioning of medium and culturing at high cell densities (reviewed in Ref. 11). The standard conditions for our studies (RPMI 10/5) allowed a consistent doubling time of 40 to 45 h regardless of plating density (from 0.25 to 4 × 10⁶ cells/3.5-cm well). At all densities, cells showed no signs of differentiation in the absence of NGF.

We next gathered baseline data on the effect of NGF on PC12 cell proliferation. NGF at 100 ng/ml (0.71 nM) induced neurite formation within several hours of exposure (data not shown). At 10⁶ cells/3.5-cm culture well, there was no discernible effect of NGF on cell number until the fifth day in culture. However, this decrease in rate of proliferation in the presence of NGF occurred earlier when cells were plated at higher density.

Although no effect of NGF on cell number was seen until day 5 at a plating density of 10⁵ cells/3.5-cm well, DNA synthesis was affected much earlier. With 24 h of exposure to 100 ng/ml NGF, DNA synthesis as measured by incorporation of [3H]thymidine showed a 50% decrease (data not shown).

Effects of NGF on PTPase Activity.

Initial studies on the effects of 1 to 5 days' exposure to 100 ng/ml NGF revealed a 2- to 4-fold increase in total lysate PTPase activity when expressed per unit cell number (Fig. 1, top). The increase in PTPase activity per unit protein was approximately 2-fold (Fig. 1, bottom). The dose-response curve for NGF-induced PTPase activation at 3 days (Fig. 2) showed a half-maximal effect at approximately 50 ng/
ml (0.35 nm). The PTPase activity measurements shown in Figs. 1 and 2 used P-Tyr-RCML, a polyacidic substrate. In comparison, PTPase activities measured with the polybasic substrate, P-Tyr-MBP, were 25% of those measured with P-Tyr-RCML. However, NGF did stimulate an approximately 2-fold increase in PTPase activity measured with P-Tyr-MBP (data not shown).

Since PC12 cell proliferation may be affected by cell density (11), we considered it essential to ascertain whether PTPase activation was actually a function of altered cell density. To study this, cells were plated at varying densities in the absence and presence of 100 ng/ml NGF. In the absence of NGF, cell density had no effect on PTPase activity per unit protein (Fig. 3) or per unit cell number (data not shown). Across the range of cell densities studied, PTPase activity was consistently higher in the presence of NGF. As noted earlier, NGF-treated cells plated at higher densities showed a more profound decrease in rate of growth, leading to attainment of lower final cell counts. The cells at highest density may have shown an attenuated PTPase response to NGF. Nonetheless, this experiment indicated that NGF-induced increases in PTPase activity were not secondary to altered cell density.

**Characteristics of PC12 Cell PTPases.** As noted in “Materials and Methods,” we measured PTPase activity in a neutral buffer and in the presence of EDTA. Under these conditions, alkaline phosphatase is inactive (12). The specificity of our PTPase measurements for protein phosphatases was further supported by sensitivity of the activities with P-Tyr-RCML and P-Tyr-MBP to micromolar concentrations of vanadate and zinc (data not shown).

To ascertain whether a single PTPase or multiple PTPases accounted for the response to NGF, lysates from control and NGF-treated cells were subjected to HPLC size exclusion chromatography (Fig. 4). Activities measured with P-Tyr-RCML (top) and P-Tyr-MBP (bottom) showed three peaks of activity corresponding to 550, 300, and 60 kDa. NGF had a relatively uniform effect on all three peaks, leading to a 3- to 4-fold increase in activity. The PTPase activity with P-Tyr-MBP, compared to activity with P-Tyr-RCML, declined as the apparent size of the PTPase decreased.

**Effect of NGF on the Proliferative Effect of EGF in PC12 Cells.** Based on the activation of PTPases associated with NGF-mediated growth inhibition, we formulated the hypothesis that PTPase activation in response to NGF leads to attenuation of the proliferative effect of other growth factors. To test this hypothesis, PC12 cells were cultured in the presence of 2% horse serum and 1% fetal bovine serum. The lower serum concentrations were used to slow growth and augment the response to a known mitogen for these cells, EGF. DNA synthesis, as measured by incorporation of [3H]thymidine (Fig. 5), was inhibited in 24 h by 100 ng/ml NGF. EGF alone (100 ng/ml, approximately 17 nM) resulted in a 25% increase in DNA synthesis. However, simultaneous addition of NGF
and EGF for 24 h revealed inhibition of DNA synthesis identical to that seen with NGF alone.

**Discussion**

Our studies show that NGF-induced differentiation in PC12 cells was accompanied by a dose-dependent increase in PTPase activity. This effect was independent of changes in cell density. Half-maximal PTPase activation was seen at a concentration of 0.35 nm. This is consistent with the concentration dependence for NGF-induced differentiation* and the affinity of NGF for the cell surface NGF receptor present in PC12 cells (13). Size exclusion chromatography of cell lysates indicated that at least three PTPases were present in undifferentiated PC12 cells. NGF treatment led to a generalized increase in all three PTPase activity peaks. Although post-homogenization proteolysis may have been involved in the multiplicity of PTPase activity peaks, no activity was detected at 20 to 40 kDa. PTPase catalytic fragments which result from proteolysis of high- and low-molecular-weight PTPases can be expected to elute at approximately 30 kDa (14).

The relationship between NGF-induced growth arrest in PC12 cells and its action via the protein tyrosine kinase trk (15) is contrary to the paradigm which associates tyrosine kinase activity with stimulation of cell growth (16). The latter association pertains to the actions of numerous growth factor receptor tyrosine kinases and nonreceptor, protooncogene tyrosine kinases. The protein tyrosine kinase inhibitor K252a blocks NGF-induced differentiation of PC12 cells (17), consistent with transmission of a growth-inhibitory signal by trk via tyrosine phosphorylation. However, in other cell systems, protein tyrosine kinase inhibitors induce differentiation and decreased growth. For example, herbimycin A causes differentiation of mouse embryonal carcinoma cells and mouse erythroleukemia cells (18). The protein tyrosine kinase inhibitors ST638 and Genistein induce terminal differentiation of mouse erythroleukemia cells when used in combination with agents that block DNA replication (19). Interestingly, staurosporine, another tyrosine kinase inhibitor, has a biphasic effect on PC12h cells (20). At low concentrations, staurosporine inhibits NGF-induced neurite formation. However, at higher concentrations, it is sufficient to induce neurite outgrowth in the absence of NGF. The variable effects of tyrosine kinase inhibitors in different cell types are consistent with in-

* D. Zachor and I. Ocrant, unpublished observations.
volvement of multiple protein tyrosine kinases in distinct signal transition pathways.

Growth factors which function as physiological activators of protein tyrosine phosphorylation also induce divergent responses in PC12 cells (reviewed in Ref. 5). Most notably, NGF and fibroblast growth factor, both of which lead to protein tyrosine phosphorylation, induce differentiation in association with growth arrest. In contrast, EGF, via its receptor tyrosine kinase, promotes PC12 cell proliferation. Since tyrosine kinase-mediated signal transduction in PC12 cells can give rise to either growth inhibition or growth stimulation, the action of a PTPase inhibitor, such as vanadate, is difficult to predict. It is possible that the NGF-induced activation of PTPases that we have observed may impinge on the actions of growth-stimulating factors, thus contributing to NGF-induced growth arrest. This hypothesis is consistent with the finding that PC12 cells exposed to NGF and EGF simultaneously for 1 day were insensitive to the mitogenic effect of EGF.

The association between cell differentiation, with its attendant decrease in proliferation, and PTPase activation may be a general phenomenon. Frank and Sartorelli (21) have shown that the granulocytic differentiation of HL60 cells in response to dimethyl sulfoxide or phorbol esters is accompanied by increased PTPase activity and a net decrease in protein tyrosine phosphorylation. In subsequent studies (22), they showed that PTPase activation is related to differentiation but not growth arrest. In contrast, Gruppuso et al. (23), working with human keratinocytes, showed that growth arrest in response to transforming growth factor β was associated with an increase in PTPase activity, whereas differentiation by transform-

**Materials and Methods**

**Materials.** 7S NGF was purchased from Collaborative Research Inc. (Bedford, MA), RPMI 1640, trypsin-EDTA (1X = 0.25% trypsin-1 mM EDTA), penicillin-streptomycin, horse serum, and fetal bovine serum were obtained from Gibco Laboratories (Grand Island, NY). Collagen (Vitrogen) for coating culture flasks and plates was purchased from the Collagen Corp. (Palo Alto, CA). Phenylmethylsulfonyl fluoride from Boehringer-Mannheim Biochemicals (Indianapolis, IN) was diluted to a concentration of 2.5% in 2-propanol. Aprotinin (Trasylo1) was from Boehringer-Mannheim, and leupeptin was from Sigma Chemical Co. (St. Louis, MO). [3H]Thymidine (15 Ci/mmole) was obtained from Dupont (NEN Products, Boston, MA).

**Cell Culture.** PC12 cells were obtained from the American Type Culture Collection (Rockville, MD). Low-passage cells were maintained in culture on 150-cm² polystyrene flasks (Science Products, Corning, NY) coated with Vitrogen in RPMI 1640 (pH 7.4), supplemented with 10% horse serum, 5% fetal bovine serum, 1% glutamine, 100 units/ml penicillin, and 100 µg/ml streptomycin (RPMI 10/5) at 37°C in a saturated water-atomosphere of 95% air and 7.5% CO₂. Where noted, serum concentrations were changed, whereas other additives were kept constant. Cells were plated on Vitrogen-coated, 6-well polystyrene plates (Becton Dickinson Labware, Lincoln Park, NJ) at a density of 10⁵ cells/3.5-cm² well except where noted. After attachment for 24 h, cells were washed twice with serum-free RPMI, and medium was replaced with RPMI with serum (concentrations as noted), with or without NGF. Plating efficiency was nearly 100% based on cell counts at the time of the initial medium change. Cells were cultured up to 5 days before harvesting. Media were changed every 3 days.

**Preparation of Cell Lysates for Phosphatase Assays.** Experiments were terminated by aspirating the media and washing the cells twice with phosphate-buffered saline. Cells were harvested in the presence of 0.5 ml lysis buffer (50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.4-2% Triton X-100-10 µg/ml leupeptin-10 µg/ml aprotinin-0.0025% phenylmethylsulfonyl fluoride). Plates were scraped with a rubber policeman to ensure complete lysis. The lysate was centrifuged at 12,000 x g for 3 min. The supernatant was saved at −70°C as three 50-ml aliquots diluted 1:2 with PTPase assay buffer (25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.4-1 mM EDTA-30 mM 2-mercaptoethanol-0.1 mg/ml bovine serum albumin). The remain-

![Graph](image-url)
order of the supernatant was stored undiluted for protein determination.

**Measurement of PTPase Activity.** PTPase activity was measured as the release of $^{32}$P, from substrates phosphorylated on tyrosine using partially purified human placental insulin receptors (25). The substrates used were P-Tyr-RCML and P-Tyr-MBP. Substrates were present at a final P-Tyr concentration of 1 μM. The assay relies on the release of $^{32}$P, from phosphorylated substrate and separation from nonreacted substrate by trichloroacetic acid precipitation (25).

**Other Methods.** Lysate protein content was measured using the bicinchoninic acid method (Pierce, Rockford, IL). Cell density was measured by detachment of cells with trypsin-EDTA (1 ml/3.5-cm well), followed by determination of cell number in a hemocytometer. Cell proliferation was also measured as incorporation of $[^3\text{H}]$thymidine into trichloroacetic acid-insoluble material (26).

**Acknowledgments**
We thank Charles T. Fay for his assistance in culturing the PC12 cells.

**References**