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Association of a Purine-Analogue-Sensitive Protein Kinase Activity with p75 Nerve Growth Factor Receptors

Cinzia Volonté,* Alonzo H. Ross,† and Lloyd A. Greene*

*Department of Pathology and Center for Neurobiology and Behavior, College of Physicians and Surgeons of Columbia University, New York, New York 10032; and †Worcester Foundation for Experimental Biology, Shrewsbury, Massachusetts 01545

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Purine analogues are protein kinase inhibitors, and they block with varying potency and specificity certain of the biological actions of nerve growth factor (NGF). The analogue 6-thioguanine (6-TG) has been shown to inhibit with high specificity protein kinase N (PKN), a serine/threonine protein kinase activated by NGF in several cellular systems. In the present work, immunoprecipitates of p75 NGF receptors from PC12 cells (+/− NGF treatment) were assayed for protein kinase activity using the substrate myelin basic protein under phosphorylating conditions optimal for PKN and in the presence or absence of purine analogues. An NGF-inducible activity was detected, and ~80% was inhibited by purine analogues. This activity was maximally stimulated by NGF within 5–10 min, partially decreased by 60 min, and returned to basal levels after 15 h of NGF treatment. The analogue 6-TG inhibited the NGF-inducible p75-associated kinase activity with an IC_{50} in the range of 15–35 μM. In mutant PC12 nnr-5 cells that lack the Trk NGF receptor, the purine-analogue-sensitive p75-associated kinase activity was not inducible by NGF. In normal PC12 cells, cyclic AMP analogues and epidermal growth factor failed to induce the same activity. Application of either 2-aminopurine or 6-TG to intact cells only slightly inhibit the NGF-dependent induction of the purine-analogue-inhibited p75-associated kinase activity. This activity shares many similarities but also displays some significant differences with cytosolic PKN. Our findings therefore indicate the association of a purine-analogue-sensitive protein kinase with p75 NGF receptors.

INTRODUCTION

Despite the increasing number of growth and differentiative agents that have been discovered and characterized, the sequence of molecular events that occurs after these agents bind to their specific receptors is just beginning to become known. Receptor-associated molecules seem to play important roles in signal transduction mechanisms, and their identification is therefore crucial for understanding the events triggered by ligand-receptor interaction.

Nerve growth factor (NGF)[1] (Levi-Montalcini, 1987) affects the survival, development, and differentiation of sympathetic as well as certain sensory and central nervous system neurons (Levi-Montalcini and Angeletti, 1968; Thoenen *et al.*, 1987; Whittemore and Seiger, 1987; Barde, 1989). NGF binds to two distinct receptor molecules on responsive cells: gp140 Trk and p75 (reviewed by Bothwell, 1991; Ross, 1991; Meakin and Shooter, 1992). The product of the proto-oncogene *trk*, $gp140^{prototrk}$, is a tyrosine kinase that has been identified as a signal-transducing and functional receptor for NGF

[1] Abbreviations used: cAMP, cyclic AMP; CPTcAMP, 5-chlorophenylthio-cAMP; EGF, epidermal growth factor; EGTA, ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; ERK, extracellular-signal regulated kinase; MBP, myelin basic protein; NGF, nerve growth factor; PAGE, polyacrylamide gel electrophoresis; PKN, protein kinase N; PMSF, phenylmethylsulfonyl fluoride; 6-TG, 6-thioguanine; 6-MMPR, 6-methylmercaptopurine riboside; 2-AP, 2-aminopurine; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane.

(Kaplan *et al.*, 1991; Klein *et al.*, 1991; Loeb *et al.*, 1991). The role of the p75 NGF receptor (Johnson *et al.*, 1986; Radeke *et al.*, 1987) in signal transduction is less clear. Some evidence would suggest that binding of NGF to p75 is not required for biological activity (Drinkwater *et al.*, 1992; Ibanez *et al.*, 1992), whereas other findings suggest that p75 plays a role in high affinity NGF binding and in signal transduction (Hempstead *et al.*, 1989, 1991; Berg *et al.*, 1991; Yan *et al.*, 1991). To date, there has been no physical evidence that p75 and Trk directly interact (Hosang and Shooter, 1985; Meakin and Shooter, 1991; Radeke and Feinstein, 1991; Meakin *et al.*, 1992).

Biological effects of NGF have been documented widely in the clonal rat pheochromocytoma cell line PC12 (Greene and Tischler, 1976), and consequently this system has served as a paradigm for studying the NGF mechanism of action (reviewed by Levi and Alemá, 1991). Studies of NGF receptors in PC12 cells have revealed at least several molecules that appear to specifically associate with Trk (Vetter *et al.*, 1991; Loeb *et al.*, 1992; Volonté, Loeb, and Greene, unpublished data). Several proteins also have been reported to coprecipitate with PC12 cell p75, including a serine kinase activity that phosphorylates several species present in the immunocomplex and that does not appear to be NGF regulated (Ohmichi *et al.*, 1991).

We recently described a serine/threonine protein kinase activity that coimmunoprecipitates with Trk (Volonté, Loeb, and Greene, unpublished data). This activity has properties similar to protein kinase N (PKN) (Rowland *et al.*, 1987; Rowland-Gagné and Greene, 1990), a basic 45- to 47-kDa protein kinase (Volonté and Greene, 1992b) that is activated by NGF and other factors in PC12 cells and other cell types (Blenis and Erikson, 1986; Rowland *et al.*, 1987; Volonté and Greene, 1990a). A feature of both the Trk-associated and soluble activities is their inhibition by purine analogues. One such analogue, 2-aminopurine (2-AP), appears to block several protein kinases in addition to PKN (Volonté and Greene, 1992a). In contrast, the analogue 6-thioguanine (6-TG) has thus far proved to be a selective and potent inhibitor of PKN activity (Volonté and Greene, 1992a). Because of their in vitro actions on PKN, purine analogues have been administered to PC12 cells and neurons. Significantly, the analogues suppress some actions of NGF but not others, thus indicating that PKN could play a role in certain, but not all, biological responses to NGF (Volonté *et al.*, 1989; Greene *et al.*, 1990; Volonté and Greene, 1990a,b, 1992a; Batistatou *et al.*, 1992).

In the present work we investigated whether there is association in PC12 cells between p75 NGF receptors and a purine-analogue-sensitive protein kinase activity. Moreover, we determined whether such an activity is specifically regulated by NGF.

MATERIALS AND METHODS

Materials

[γ-^{32}P] ATP (3000 Ci/mmol) was purchased from New England Nuclear (Boston, MA); Protein A Sepharose CL-4B was from Pharmacia (Piscataway, NJ); 6-TG, 2-AP, 6-methylmercaptopurine riboside, ATP, and myelin basic protein (MBP) were purchased from Sigma Biochemicals (St. Louis, MO); Minifold II Slot Blotter Apparatus and nitrocellulose (BA85-SB, 0.45 μm pore) were from Schleicher & Schuell (Keene, NH). Anti-gp140^{prototrk} antiserum 443-BO (Soppet *et al.*, 1991) was a gift from Dr. L.F. Parada (Frederick Cancer Research Center, Frederick, MD) and anti-phosphotyrosine antibody 4G10 (Kaplan *et al.*, 1990) was a gift from Dr. D.R. Kaplan (Frederick Cancer Research Center, Frederick, MD). The rabbit polyclonal antiserum, which recognizes the intracellular portion of p75 NGF receptors (Ohmichi *et al.*, 1991), was a kind gift from Dr. M.V. Chao (Cornell University Medical College, NY).

Cell Culture

PC12 cells were cultured as previously described on collagen-coated culture dishes (150-mm diameter) in RPMI 1640 medium supplemented with 10% heat inactivated horse serum and 5% fetal bovine serum (Greene and Tischler, 1976). NGF, purified from mouse submaxillary glands (Mobley *et al.*, 1972), was directly added to the cultures for 5 min, unless otherwise specified, at a final concentration of 100 ng/ml. The cultures were washed twice with ice-cold phosphate-buffered saline and then quick-frozen in liquid nitrogen and stored at −80°C until use.

Immunoprecipitation of p75 and p75-Associated Proteins

Cells were lysed for immunoprecipitation in 750 μl of lysis buffer (LB) containing 50 mM tris(hydroxymethyl)aminomethane (Tris) (pH 7.5), 10% glycerol, 1% Triton X-100, 150 mM NaCl, 100 mM NaF, 5 μM $ZnCl_2$, 1 mM Na_3VO_4, 10 mM ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA), 2 mM phenylmethylsulfonyl fluoride (PMSF), 200 KIU/ml aprotinin, and 1 μg/ml leupeptin. After extraction on ice for 10 min, lysates were centrifuged at 100 000 × *g* for 15 min at 4°C. Supernatants were reserved and normalized for protein concentration. Equal amounts of protein were incubated for 2 h at 4°C with anti-rat p75 NGF receptor mouse monoclonal ascites (192) (Chandler *et al.*, 1984) at a dilution of 1:700. Protein A Sepharose, prepared as suggested by Upstate Biotechnology (Lake Placid, NY), was added to the lysate for 1 h. The Protein A Sepharose/antiserum complex was pelleted at 4°C in a microfuge, and the pellets were washed three times (1 ml/wash) with TNTG (20 mM Tris, pH 7.5, 150 mM NaCl, 0.1% Triton X-100, 10% glycerol). In some cases, blanks were prepared by omitting the primary antibody.

Assay of p75 NGF Receptor-Associated Protein Kinase Activity

After immunoprecipitation, the immune complexes were suspended in 100–250 μl of ice-cold phosphorylation buffer (50 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, pH 7.4, 2 mM EGTA, 10 mM NaF, 10 mM $MnCl_2$, 2 mM PMSF, 100 kIU/ml Trasylol, 5 μg/ml leupeptin), and 10 μl were used for phosphorylation assays. These were performed in phosphorylation buffer (100 μl final volume) using 10 μg of MBP as substrate and 5 μCi of [γ-^{32}P] ATP (3000 Ci/mmol) in a final ATP concentration of 20 μM. The mixture was incubated for 15 min at 37°C, and the phosphorylating reactions were terminated by the addition of 40 μl of 4×-electrophoretic sodium dodecyl sulfate (SDS) containing sample buffer. Aliquots were boiled and then either subjected to SDS-polyacrylamide gel electrophoresis (PAGE) (Laemmli, 1970) and autoradiography or adsorbed under vacuum onto nitrocellulose, using a slot-blotter apparatus, to separate

the phosphorylated substrates from free [γ-^{32}P] ATP (Volonté *et al.*, 1992). In the latter case, single slots were excised and assessed for radioactivity by liquid scintillation counting. Blanks were determined from samples incubated in parallel without the substrate and without the immune complex, all as previously described (Volonté *et al.*, 1992).

Protein Determination

Protein concentrations were determined by the method of Bradford (1976), using reagents and protocols purchased from Bio-Rad Laboratories (Richmond, CA) and with bovine serum albumin as standard.

RESULTS

PC12 cells cultured for different times with or without NGF were solubilized in a Triton-containing buffer and subjected to immunoprecipitation with a monoclonal antibody that recognizes the rat p75 NGF receptor. The immunoprecipitates were extensively washed and then tested for protein kinase activity using MBP as an exogenous substrate. The phosphorylations were performed under conditions (10 mM Mn^{2+}, pH 7.4, 10 mM NaF) previously found optimal for soluble PKN activity (Volonté *et al.*, 1989). After treatment of PC12 cells with NGF for 5–10 min, a two- to threefold increase in p75-associated MBP phosphorylating activity was obtained (Figure 1). The extent of induction significantly decreased after the cells were cultured for 1 h with NGF and returned to basal levels after 15 h. Basal as well as NGF-induced activities are inhibited between 50 and 90% in the presence of 500 μM 6-TG (Figure 1, Table 1) or 10 mM 2-AP (Table 1). Because protein A is not always quantitative in recognizing mouse Ig, the immunoprecipitations were also carried out with rabbit anti mouse Ig-protein A conjugates (2 h at 4°C) or simply rabbit anti-mouse Ig (18 h at 4°C) to ensure optimal recovery of p75 and associated proteins. For this reason, the p75 antibody was also tested at dilutions as low as 1:100. In all cases, no significant differences in recovery of the p75-associated kinase activity were observed.

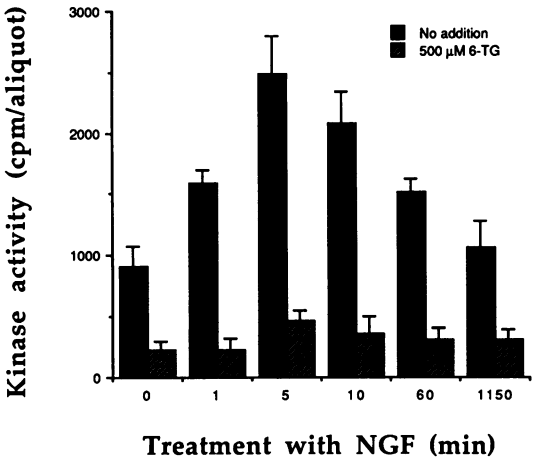

Figure 1. Time-course of NGF-dependent induction of p75-associated kinase activity: sensitivity to 6-TG. Replicate PC12 cell cultures were treated for different times in the absence or presence of 100 ng/ml NGF. Extracts were then subjected to immunoprecipitation using anti-p75 monoclonal antiserum 192, and the immunoprecipitates were incubated with MBP under phosphorylating conditions for 15 min at 37°C, with or without 500 μM 6-TG, as described in MATERIALS AND METHODS. Aliquots (50 μl) were then adsorbed under vacuum onto nitrocellulose and assessed by liquid scintillation counting for specifically incorporated radioactivity. Results are shown as average of triplicate determinations ± SEM (n = 3). Comparable results were achieved in three independent experiments.

Table 1. p75-Associated MBP phosphorylating activity: inhibition by purine analogues

	6-TG 500 μM	2-AP 10 mM	6-MMPR 100 μM
Control (n = 13)	65 ± 22	50, 94	0, 0
NGF (n = 18)	80 ± 13	95, 95	0, 10

PC12 cells were incubated for 5 min in the absence or presence of 100 ng/ml NGF. p75-Associated proteins were immunoprecipitated, and MBP kinase activity was tested as described in MATERIALS AND METHODS. Results are shown as means ± SEM (n > 2) or as results of two independent experiments.

To test whether the association between p75 and the purine-analogue-sensitive MBP phosphorylating activity was specific, several different controls were performed. First, to test nonspecific recognition of rat proteins, the monoclonal antibodies were preabsorbed with rat serum for 30 min at 37°C before use. No decrease in coprecipitated kinase activity was observed. In addition, immunoprecipitation blanks prepared from NGF-treated cells and in which the primary antiserum was omitted, possessed only ~10–15% of the MBP phosphorylating activity obtained with anti-p75 antibody. Finally, a rabbit polyclonal antiserum that recognizes the intracellular portion of p75 NGF receptors (Ohmichi *et al.*, 1991) was also found to immunoprecipitate the 6-TG sensitive NGF-activatable protein kinase activity from PC12 cell extracts.

We recently found that a purine-analogue-sensitive MBP phosphorylating activity associates and coimmunoprecipitates with Trk NGF receptors (Volonté *et al.*, unpublished data). To evaluate the possibility that the association of purine-analogue-sensitive MBP phosphorylating activity with p75 was due to coimmunoprecipitation of the Trk NGF receptor, the p75 immunoprecipitates from NGF-treated cultures were resolved by SDS-PAGE, western blotted to nitrocellulose, and probed with anti-gp140^{prototrk} antiserum 443-BO (Soppet *et al.*, 1991) or anti-phosphotyrosine antibody 4G10 (Kaplan *et al.*, 1990). In both cases, the presence of gp140^{prototrk} was not detected.

To further test the specificity of the above effects, we used a mutant PC12 cell subline (PC12 nnr5) that lacks high affinity NGF binding, the Trk NGF receptor, and

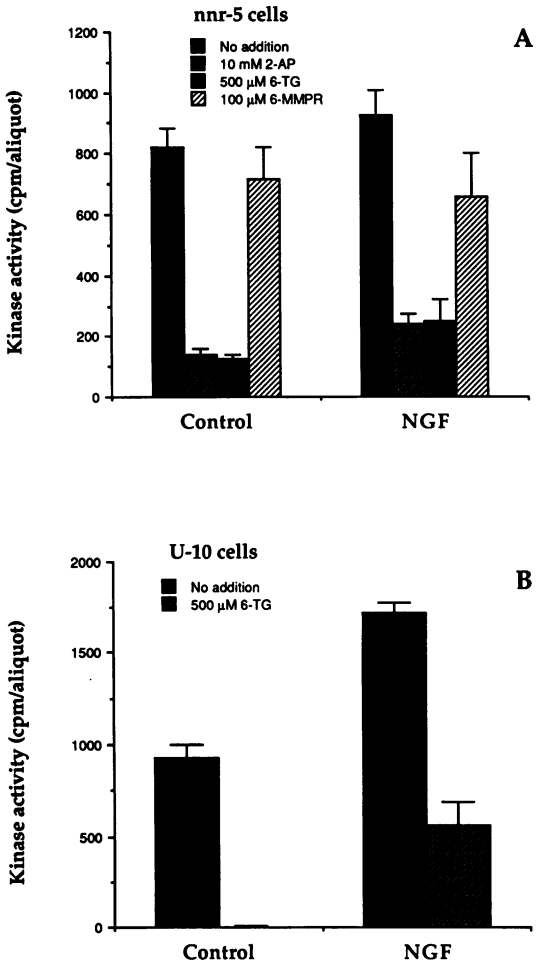

Figure 2. p75-Associated protein kinase activity from different cell lines. In (A) replicate PC12 nnr-5 cell cultures and in (B) PC12 U-10 cell cultures were treated for 5 min in the presence or absence of 100 ng/ml NGF. Extracts were then subjected to immunoprecipitation using anti-p75 monoclonal antiserum 192, and the immunoprecipitates were incubated with MBP (± the indicated purine analogues) under phosphorylating conditions for 15 min at 37°C as described in MATERIALS AND METHODS. Aliquots (50 μl) were then adsorbed under vacuum onto nitrocellulose and assessed by liquid scintillation counting for specifically incorporated radioactivity. Results are shown as average of triplicate determinations ± SEM (n = 3). Comparable results were achieved in two independent experiments.

all responsiveness to the factor (Green *et al.*, 1986; Loeb *et al.*, 1991). These cells, however, express p75 (Green *et al.*, 1986; Loeb *et al.*, 1991). As with PC12 cells, nnr5 cells showed a basal level of p75-associated MBP kinase activity that was inhibitable by the purine analogues 2-AP and 6-TG (Figure 2A). However, in contrast to PC12 cells, there was no increase in activity in response to NGF. In addition, in the PC12 derivative U10 cell line in which NGF elicits a mitogenic rather than differentiative response (Greene and Rukenstein, 1981; Burstein and Greene, 1982), we observed a twofold NGF-dependent induction of the kinase activity associated with p75 NGF receptor and inhibition by 6-TG (Figure 2B). This suggests that the PKN-like activity associates with p75 in an NGF-dependent manner irrespective of whether NGF binding elicits a proliferative or differentiative response.

We next tested the specificity of NGF in stimulating p75-associated MBP kinase activity. PC12 cells were treated with the cyclic AMP (cAMP) analogue 5-chlorophenylthio-cAMP (CPTcAMP). This permeant cAMP derivative leads to rapid activation of at least several protein kinase activities in PC12 cells, among which is a soluble PKN-like activity (Blenis and Erikson, 1986; Rowland *et al.*, 1987; Rowland-Gagné and Greene, 1990). The data in Figure 3 show that in contrast to NGF, CPTcAMP does not stimulate p75-associated MBP kinase activity. Epidermal growth factor (EGF), which stimulates cytoplasmic PKN activity, also failed to induce the p75-associated kinase (Figure 3). Aside from addressing specificity, these data further indicate that the association between p75 and purine-analogue-sensitive MBP phosphorylating activity is not due to nonspecific coimmunoprecipitation of soluble PKN with low affinity NGF receptors.

To further analyze the p75-associated kinase activity, MBP was phosphorylated in the presence of immunoprecipitates from both control and NGF-treated PC12 cells and [γ-^{32}P] ATP and then subjected to phosphoamino acid analysis. In both control and NGF-treated samples, phosphorylation was detected on threonine and serine but not on tyrosine. Only phosphorylation on threonine was increased in samples from NGF-treated cells, and 500 μM 6-TG inhibited phosphorylation on both serine and threonine (Figure 4). Additional phosphorylation experiments were carried out with p75 immunoprecipitates from NGF-treated PC12

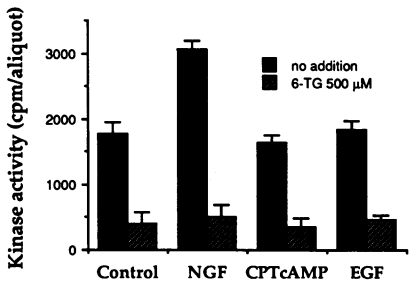

Figure 3. CPTcAMP and EGF do not stimulate p75-associated protein kinase activity. Replicate PC12 cell cultures were treated for 5 min in the presence of either 100 ng/ml NGF, 500 μM CPTcAMP, or 2.6 ng/ml EGF. Extracts were then subjected to immunoprecipitation using anti-p75 monoclonal antiserum 192, and the immunoprecipitates were incubated with MBP (±500 μM 6-TG) under phosphorylating conditions for 15 min at 37°C as described in MATERIALS AND METHODS. Aliquots (50 μl) were then adsorbed under vacuum onto nitrocellulose and assessed by liquid scintillation counting for specifically incorporated radioactivity. Results are shown as average of triplicate determinations ± SEM (n = 3). Comparable results were achieved in two independent experiments.

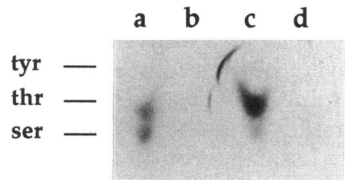

Figure 4. Phosphoamino acid analysis of MBP phosphorylated by p75-immunoprecipitates in the presence or absence of 6-TG. MBP was used as the in vitro substrate for p75-associated kinase activity immunoprecipitated from replicate control and NGF-treated PC12 cultures under conditions described in MATERIALS AND METHODS. The phosphorylation of MBP was performed in the absence or presence of 500 μM 6-TG. Triplicate samples from each experimental condition were pooled, and phosphoamino acids were prepared and separated by thin-layer chromatography as described by Cooper *et al.* (1983) and Muñoz and Marshall (1990). All recovered material from each sample was evaluated. Lanes a and b, MBP-derived phosphoamino acids phosphorylated by p75-immunoprecipitates from control PC12 cultures. Lanes c and d, MBP-derived phosphoamino acids phosphorylated by the p75-immunoprecipitates from NGF-treated PC12 cultures. In lanes b and d the in vitro phosphorylation of MBP was performed in the presence of 500 μM 6-TG. The positions of unlabeled markers (phosphoserine, phosphothreonine, and phosphotyrosine) are indicated.

cells, but with histone HF1, fos and jun proteins as potential substrates. Each of these is an effective substrate for soluble NGF-stimulated PKN (Volonté and Greene, 1992b). These proteins all served as excellent exogenous substrates for the p75-associated activity and phosphorylation was in each case inhibited by 6-TG.

To characterize the 6-TG sensitivity of the p75-associated kinase activity, phosphorylation of MBP was performed in the presence of identical aliquots of p75 immunoprecipitates from NGF-treated PC12 cells and different concentrations of this purine analogue. The data in Figure 5 indicate that 6-TG inhibits ~90% of the NGF-inducible p75-associated kinase activity with an apparent IC_{50} in the range of 15–20 μM. Over a larger number of experiments (Table 1), 500 μM 6-TG inhibited an average of 80 ± 13% (mean ± SD) of p75-associated MBP phosphorylating activity from NGF-treated cells, and the IC_{50} ranged between 15 and 35 μM (n = 3). Prior studies indicated that 6-TG inhibits soluble NGF-stimulated PKN activity with an apparent IC_{50} of 5–10 μM (Volonté *et al.*, 1989). The most potent specific inhibitor of soluble NGF-stimulated PKN presently known is 6-methylmercaptopurine riboside (6-MMPR), which blocks this activity with an apparent IC_{50} of ~10 nM (Volonté and Greene, 1992a). However, in contrast to 6-TG and 2-AP, 6-MMPR does not significantly inhibit either basal or NGF-stimulated p75-associated kinase activity from PC12 (Table 1) or nnr5 cells (Figure 2A).

Previous work established that application of 2-AP to intact PC12 cells blocks the in situ activation of soluble PKN by NGF (Volonté *et al.*, 1989). 6-TG, in contrast, does not have this effect (Volonté *et al.*, 1989). In the present experiments, PC12 cells were pretreated for 20 min with either 10 mM 2-AP or 500 μM 6-TG. The cultures were subsequently exposed to NGF for 5 min (in the continued presence of 2-AP or 6-TG) and then the cellular extracts were used for immunoprecipitation of p75. Figure 6 shows that 6-TG does not inhibit the induction of purine-sensitive p75-associated MBP phosphorylating activity. In contrast to cytosolic PKN, in which case 2-AP totally prevents NGF-dependent activation (Volonté *et al.*, 1989), the induction by NGF of p75-associated kinase activity is only slightly reduced by 2-AP. When 6-TG or 2-AP were added directly to the kinase reactions, however, they effectively inhibited both basal and NGF-induced activities.

DISCUSSION

It has been shown recently that NGF binds to two different receptors molecules on responsive cells: p140 Trk, a tyrosine kinase receptor that is the product of the proto-oncogene *trk*, and p75 (reviewed by Bothwell, 1991; Ross, 1991; Meakin and Shooter, 1992). In several cellular systems, serine/threonine kinases have been shown to be activated by, or to associate with, growth factor receptor-tyrosine kinases complexes (Morrison *et*

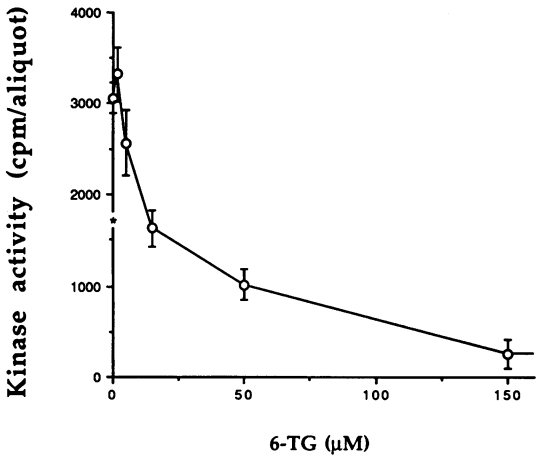

Figure 5. 6-TG inhibits p75-associated protein kinase activity: dose-response effects. Replicate PC12 cell cultures were treated for 5 min in the absence or presence of 100 ng/ml NGF. Extracts were then subjected to immunoprecipitation using anti-p75 monoclonal antiserum 192, and the immunoprecipitates were incubated with MBP under phosphorylating conditions for 15 min at 37°C in the presence of different concentrations of 6-TG, all as described in MATERIALS AND METHODS. Aliquots (50 μl) were then adsorbed under vacuum onto nitrocellulose and assessed by liquid scintillation counting for specifically incorporated radioactivity. The star on the axis represent the kinase activity of PC12 cells in the absence of NGF. Results are shown as average of triplicate determinations ± SEM (n = 3). Comparable results were achieved in three independent experiments.

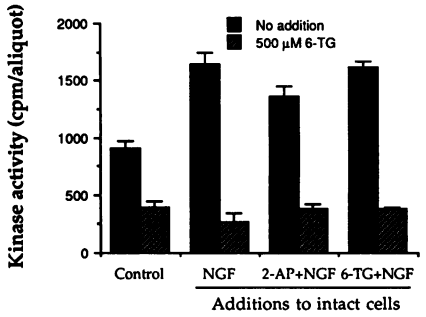

Figure 6. Purine analogues do not prevent NGF-dependent activation of p75-associated protein kinase activity when added to intact cells. Replicate PC12 cell cultures were pretreated where indicated for 20 min in the presence of 10 mM 2-AP or 500 μM 6-TG and then treated for an additional 5 min in the presence of 100 ng/ml NGF. Extracts were then subjected to immunoprecipitation using p75 antiserum 192, and the immunoprecipitates were incubated under phosphorylating conditions with MBP for 15 min at 37°C with or without 500 μM 6-TG, all as described in MATERIALS AND METHODS. Aliquots (50 μl) were then adsorbed under vacuum onto nitrocellulose and assessed by liquid scintillation counting for specifically incorporated radioactivity. Results are shown as average of triplicate determinations ± SEM (n = 3). Comparable results were achieved in two independent experiments.

al., 1989; Ettehadieh *et al.*, 1992). This is also the case for Trk NGF receptors. For example, a specific member (early response kinase [ERK]1) of the multigene ERK family of protein kinases becomes specifically associated with Trk after exposure of PC12 cells to NGF (Loeb *et al.*, 1992). We have furthermore shown that NGF enhances the level of purine-analogue-sensitive PKN-like protein kinase activity associated with Trk (Volonté, Loeb, and Greene, unpublished data).

The present work extends these findings by demonstrating the specific association of a 6-TG-sensitive protein kinase activity with low affinity p75 NGF receptors. Several observations indicate that the coimmunoprecipitation is not an artifact and is not due to nonspecific binding of soluble and purine-analogue-inhibited PKN to p75 or to coprecipitation of Trk receptors with p75. First, this activity was not decreased if the anti-p75 monoclonal antibodies used in this study were preabsorbed with rat serum and was nearly completely eliminated if the antibodies were omitted. Furthermore, a rabbit polyclonal antiserum that recognizes the intracellular portion of p75 NGF receptor (Ohmichi *et al.*, 1991) was also found to coimmunoprecipitate the purine-sensitive MBP kinase activity. In addition, in the mutant PC12nnr5 subline that lacks Trk-high affinity NGF binding and responsiveness to the factor (Green *et al.*, 1986; Loeb *et al.*, 1991), there was no NGF-dependent activation of p75-associated purine-analogue-inhibited kinase activity. Also, when PC12 cells were treated with a cAMP analogue or with EGF, which rapidly lead to activation of soluble PKN-like activity (Blenis and Erikson, 1986; Rowland *et al.*, 1987; Rowland-Gagne and Greene, 1990), the induction of p75-associated purine-analogue-sensitive activity was not observed. Moreover, the time courses for NGF-dependent activation of soluble PKN and of the p75-associated enzyme were rather different; the latter peaked at 5 min and returned to basal level by 15 h, whereas the former reaches a plateau by 5 min and remains activated for at least 12 h (Volonté *et al.*, 1989). Soluble, Trk-associated, and p75-associated MBP phosphorylating activity also differed in their sensitivity to purine analogues (see below). Finally, the presence of $gp140^{prototrk}$ was not detected in the p75 immunoprecipitates.

The kinase activity associated with the p75 NGF receptor shares a number of characteristics with soluble PKN and with purine-sensitive MBP kinase activity associated with Trk. In particular, it functions with Mn^{++} as the sole divalent cation; uses as in vitro substrates myelin basic protein, histone HF1, fos and jun proteins; phosphorylates on serine and threonine residues; is activated about two- to threefold by NGF; is rapidly activated (within 5 min) and is inhibited by 6-TG and 2-AP. The p75-associated activity also appears to have properties that distinguish it from soluble PKN and from the Trk-associated activity. Different from soluble PKN, but more similar to the Trk-associated purine-analogue-sensitive kinase activity, the enzyme coimmunoprecipitated by p75 antibodies is transiently activated by NGF and is not activated by a cAMP analogue. Also, in contrast to soluble PKN, activation of the p75-associated activity by NGF in intact cells is only slightly inhibited by exposure to 2-AP. This suggests that NGF activates the p75-associated enzyme and cytosolic PKN through distinct pathways or that soluble PKN is activated by the p75-associated enzyme. We previously found that activation of the Trk-associated activity is also not blocked by 2-AP. Another difference between soluble PKN, Trk-associated MBP kinase activity, and the p75-associated activity is sensitivity to 6-MMPR. The latter is barely inhibited by 100 μM 6-MMPR, the most potent specific inhibitor of soluble NGF-stimulated PKN presently known (IC_{50} about 10 nM) (Volonté and Greene, 1992a). For the case of Trk-associated activity, 6-MMPR was effective but with an apparent IC_{50} of 1–10 μM. In past studies, this compound failed to inhibit cAMP-activated cytosolic PKN (Volonté and Greene, 1992a), suggesting that 6-MMPR may discriminate among different forms of PKN. Finally, the three activities show different apparent sensitivity to 6-TG. For the p75-associated activity, the apparent IC_{50} was 20–35 μM, for the Trk-associated activity, 100–500 μM, and for the soluble PKN, 5–10 μM. Our past studies have indicated that soluble PKN appears to be comprised of a family of related but distinguishable forms (Volonté and Greene, 1992b). On this basis, the present findings are consistent with the possibility that soluble, Trk-associated, and p75-associated purine-analogue-sensitive protein kinase activities are distinct but related members

of the same family of enzymes. As a precedent for this, although protein kinases ERK1 and ERK2 are very closely related, only the former appears to associate with $p140^{prototrk}$ (Loeb *et al.*, 1992). Alternatively, these apparent differences in PKN-like activity could be due to alterations in structure or to accessibility of the receptor-associated molecules. Finally, we cannot rule out the possibility that all, or a portion thereof, are unrelated.

Irrespective of whether the purine-analogue-sensitive protein kinase activity associated with p75 is related to cytosolic PKN or to the Trk-associated enzyme, these findings in turn raise the issue of the significance of this interaction. One possibility is that p75 itself (Taniuchi *et al.*, 1986) or other molecules associated with p75 are substrates for this protein kinase activity and that this phosphorylation regulates their functional properties. Interaction/phosphorylation of receptor or receptors-associated enzymes by a serine/threonine kinase has been documented (Northwood *et al.*, 1991; Ohmichi *et al.*, 1991). An alternative is that the purine-analogue-sensitive kinase may be a substrate of another p75-associated activity.

Ohmichi *et al.* (1991) previously reported the presence of a serine kinase activity present in p75 immunocomplexes. This activity phosphorylated two proteins (60 and 130 kDa) also present in the complex. NGF treatment resulted in increased phosphorylation of the 130-kDa species. Because the immunocomplex did not show an NGF-dependent increase in phosphorylation of an endogenous substrate (enolase), it was suggested that NGF did not regulate the p75-associated kinase activity but rather affected interaction of the 130-kDa species with the complex. We do not presently know the relationship between this activity and the purine-sensitive kinase described here. However, one potential contrast between the two is that only the latter appears to be NGF-regulated. One interpretation consistent with the present data is that the previously described activity corresponds to the purine-insensitive activity described here. In both cases, this activity appears to be NGF unregulated and serine directed.

We have shown that NGF-promoted activation of p75-associated purine-analogue-inhibited kinase activity is dependent on the expression of Trk NGF receptors; this confirms the importance of Trk in triggering biological effects of NGF in PC12 cells. Our findings could also indicate an indirect interaction or mutual influence between Trk and p75, because both NGF receptors appear to associate with the same family of purine-analogue-inhibited protein kinases, suggesting even possible cross-talk between the two NGF receptors.

We have shown previously that application of purine analogues to cells suppresses a subset of early as well as late responses to NGF (Volonté *et al.*, 1989; Volonté and Greene, 1990a,b, 1992a; Greene *et al.*, 1990; Batistatou *et al.*, 1992). In light of the present and recent data, both p75- and Trk-associated kinase activities must be considered as potential targets for purine analogues and thus as potential elements in the NGF mechanism of action.

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