p75^{NGFR} and TrkA receptors collaborate to rapidly activate a p75^{NGFR}-associated protein kinase

Marco Canossa^{1,2,3}, Jeffery L. Twiss^{2,4},
A.Neil Verity² and Eric M. Shooter²

¹Department of Pharmacology, Bologna University, Via Imerio 48, Bologna 40126, Italy and ²Departments of Neurobiology and ³Pathology, Stanford University School of Medicine, Stanford, CA 94305-5401, USA

³Corresponding author

M.Canossa and J.L.Twiss contributed equally to this work

The role of the low affinity nerve growth factor receptor (p75^{NGFR}) in NGF-mediated signaling is not yet understood. Here we show by co-immunoprecipitation that NGF activates a protein kinase that is directly associated with p75^{NGFR} in dorsal root ganglion (DRG) cells and PC12 cells in culture. Two proteins of 120 and 104 kDa constitute the majority of this activity. In PC12 cells, TrkA activation was necessary to elicit p75^{NGFR}-associated kinase activity. Although NGF binding to p75^{NGFR} was not necessary for kinase activation, it accelerated the activation of the kinase at low NGF concentrations. Deletion analysis showed that a 43 amino acid region in the cytoplasmic domain of p75^{NGFR} was responsible for this effect. These findings show that p75^{NGFR} accelerates TrkA-mediated signaling and, in addition, demonstrate that p75^{NGFR} and TrkA collaborate to activate a previously undescribed p75^{NGFR}-associated protein kinase.

Keywords: p75^{NGFR}/phosphorylation/protein kinase/signal transduction/TrkA

immediate early gene, *c-fos* (Barker and Shooter, 1994) and the rate of maturation of the sympathoadrenal progenitor cell line, MAH (Verdi *et al.*, 1994). Although the detailed mechanism behind this collaboration is not yet known, the effect of p75^{NGFR} is most simply explained by the facilitation of the interaction of NGF with the extracellular domain of TrkA.

p75^{NGFR} may also participate in intracellular signal transduction. For instance, NGF binding to p75^{NGFR} in the absence of TrkA induces ceramide production in T9 glioma cells and cAMP in PCNA fibroblasts (Knipper *et al.*, 1993; Dobrowsky *et al.*, 1994). Moreover, p75^{NGFR} appears to signal apoptosis in the absence of NGF binding (Rabizadeh *et al.*, 1993; Barrett and Bartlett, 1994). Furthermore, a number of protein kinases are known to associate with p75^{NGFR} (Omichi *et al.*, 1991; Volonté *et al.*, 1993a,b). The activation of a p75^{NGFR}-associated protein kinase, either directly by NGF binding to p75^{NGFR} or indirectly via TrkA activation, could provide a mechanism by which p75^{NGFR} can participate in NGF signal transduction. We show here that NGF activates a previously undescribed 120/104 kDa protein kinase associated with p75^{NGFR} in dorsal root ganglion (DRG) cells and PC12 cells in culture. At low NGF concentrations, ligand binding to p75^{NGFR} results in accelerated kinase activation in PC12 cells. Only slow activation of this kinase occurs in the absence of ligand binding to p75^{NGFR} or when the ratio of p75^{NGFR} to TrkA is decreased. The high levels of activity of this protein kinase suggest that it may play an important role in p75^{NGFR}-mediated NGF signal transduction.

Introduction

Nerve growth factor (NGF), the prototype of an expanding family of neurotrophins, binds to two different receptors, TrkA and p75^{NGFR} (Chao, 1992; Meakin and Shooter, 1992; Barbacid, 1993; Snider, 1994). Activation of TrkA, a 140 kDa protein with intrinsic tyrosine kinase activity, is necessary and sufficient for NGF activity (Kaplan *et al.*, 1991; Ibáñez *et al.*, 1992; Rovelli *et al.*, 1993). The role of p75^{NGFR}, a 75 kDa membrane-spanning glycoprotein with no known intrinsic kinase activity, is more complex (Chao and Hempstead, 1995). p75^{NGFR} interacts with TrkA to enhance the rate of association of NGF to TrkA (Mahedeo *et al.*, 1994), thereby increasing its affinity for the ligand (Hempstead *et al.*, 1991; Battleman *et al.*, 1993; Benedetti *et al.*, 1993). These effects are only observed if the ratio of p75^{NGFR} to TrkA is significantly greater than one (Mahedeo *et al.*, 1994). The collaboration between p75^{NGFR} and TrkA is apparent at low concentrations of NGF where binding to p75^{NGFR} enhances the binding of NGF to TrkA and its autophosphorylation (Barker and Shooter, 1994; Verdi *et al.*, 1994), the activation of the

Results

Demonstration of p75^{NGFR}-associated protein kinase activity

To determine whether NGF activates protein kinases associated with p75^{NGFR}, dissociated rat DRG and PC12 cells in culture were treated with NGF (50 ng/ml) for 5 min and cell lysates prepared and immunoprecipitated with a monoclonal antibody (MC192) specific for the extracellular domain of rat p75^{NGFR}. Protein kinase activity that co-immunoprecipitated with p75^{NGFR} was observed in a cell-free phosphorylation assay using myelin basic protein (MBP) as substrate. As shown in Figure 1, in PC12 (lanes 1 and 2) and DRG cells (lanes 3 and 4), NGF treatment elicited a p75^{NGFR}-associated protein kinase activity. In the absence of NGF treatment, low levels of MBP phosphorylation were detected and this was also found with NGF-treated lysates in the absence of primary antibody (data not shown). These non-specific levels of MBP phosphorylation are likely to be due to low levels of non-specific binding to the agarose beads. In all experiments, however, p75^{NGFR}-associated kinase

Fig. 1. Detection of protein kinase activity associated with p75^{NGFR}. PC12 cells (lanes 1 and 2) and dissociated dorsal root ganglion (DRG) from post-natal day 9 (P9) rats in culture (lanes 3 and 4) were treated with serum-free medium (lanes 1 and 3) or NGF 50 ng/ml (lanes 2 and 4) for 5 min. p75^{NGFR} was immunoprecipitated from cell lysates using a monoclonal antibody (MC192) that recognizes its extracellular domain. A cell-free protein kinase assay was performed by incubating the immunocomplexes with MBP as substrate. The arrow indicates the electrophoretic mobility of MBP in 12% SDS-PAGE. NGF treatment in both cell types (lanes 2 and 4) induced a p75^{NGFR}-associated kinase activity as revealed by MBP phosphorylation. Equivalent amounts of protein were used for immunoprecipitation with MC192.

activity exceeded this background activity by at least 3-fold. Similar data have been reported by Omichi *et al.* (1991) and Volonté *et al.* (1993a,b) for PC12 cells. In PC12 cells, a rabbit polyclonal antiserum directed against the intracellular domain of p75^{NGFR} produced similar results (data not shown).

NGF binding to p75^{NGFR} is not required for the activation of the p75^{NGFR}-associated kinase

Two experimental paradigms show that NGF binding to p75^{NGFR} is not required for activation of its associated kinase. First, we used a PC12 cell line (pcTL) stably transfected with a chimeric receptor consisting of the extracellular domain of the human type-2 tumor necrosis factor receptor (TNFR2) fused with the transmembrane and cytoplasmic domains of rat p75^{NGFR} (TNFR2-p75) (Rovelli *et al.*, 1993). After treating pcTL cells with NGF for 5 min, wild-type p75^{NGFR} was immunoprecipitated using the MC192 antibody and chimeric TNFR2-p75 was immunoprecipitated using a monoclonal antibody (utr-4) specific for the extracellular domain of the human TNFR2 receptor. NGF-dependent protein kinase activity was detected in both MC192 and utr-4 immunocomplexes (Figure 2A, lanes 3 and 4 and 5 and 6, respectively). Since NGF does not bind to the human TNFR2 receptor (Rovelli *et al.*, 1993), the induction of protein kinase activity associated with the chimeric TNFR2-p75 receptor can occur in the absence of direct NGF binding to this receptor. Second, treatment with a mutated NGF (NGF^{3T}, 50 ng/ml; Ibáñez *et al.*, 1992) which binds to TrkA but not to p75^{NGFR} yielded levels of p75^{NGFR}-associated protein kinase activity that were comparable with those elicited by wild-type NGF (Figure 2B). Both experiments imply the involvement of TrkA in mediating p75^{NGFR}-associated kinase activity.

Binding of NGF to TrkA is required for p75^{NGFR}-associated kinase activation

To determine whether NGF-mediated TrkA tyrosine kinase activity is necessary for activation of the p75^{NGFR}-associated protein kinase, we first used a variant PC12 cell line that expresses p75^{NGFR} but lacks TrkA receptors (nnr5; Green *et al.*, 1986). NGF treatment of nnr5 cells did not

Fig. 2. Role of p75^{NGFR} and TrkA in p75^{NGFR}-associated protein kinase activity. (A) PC12, pcTL and nnr5 cells were treated with 50 ng/ml NGF for 5 min (lanes 2, 4, 6 and 8). Untreated cells were included as controls (lanes 1, 3, 5 and 7). Equivalent amounts of protein were used for immunoprecipitation with either MC192 (lanes 1–4, 7 and 8) or utr-4 antibodies (lanes 5 and 6). A protein kinase assay was performed by incubating the immunocomplexes with MBP as substrate. NGF treatment of PC12 or pcTL cells resulted in the induction of MBP kinase activity co-immunoprecipitating with p75^{NGFR} (lanes 2 and 4 respectively). Proteins associated with TNFR2-p75 chimeric receptors showed NGF-dependent kinase activity (lane 6). NGF treatment of nnr5 cells which express p75^{NGFR} but not TrkA showed no induction of p75^{NGFR}-associated protein kinase activity in response to NGF (lane 8). (B) pcTA, a PC12 cell line expressing the chimeric receptor TNFR2-TrkA, was treated with 50 ng/ml of NGF, TNF or NGF^{3T} for 5 min. p75^{NGFR} was immunoprecipitated with MC192 antibody and the MBP kinase assay was performed (top). Regardless of ligand binding to p75^{NGFR}, increased kinase activity was detected after activation of either TrkA by NGF and NGF^{3T} or TNFR2-TrkA by TNF (lanes 2–4). Aliquots of each sample showed comparable levels of p75^{NGFR} protein by Western blot (bottom).

elicit p75^{NGFR}-associated protein kinase activity (Figure 2A, lanes 7 and 8). Second, PC12 cells expressing TNFR2-TrkA (pcTK cells) chimeric receptors in which the TrkA tyrosine kinase domain was rendered inactive by a point mutation (Rovelli *et al.*, 1993; Canossa *et al.*, 1994) were used. TNF treatment of pcTK cells did not induce p75^{NGFR}-associated kinase activity (data not shown). When we used a PC12 cell line (pcTA) that is stably transfected with a chimeric receptor consisting of the extracellular domain of the human TNFR2 and the transmembrane and cytoplasmic domains of rat TrkA (Rovelli *et al.*, 1993; Canossa *et al.*, 1994), however, human TNF or mouse NGF treatment at the concentration of 50 ng/ml induced similar levels of p75^{NGFR}-associated protein kinase activity, despite the absence of ligand binding to p75^{NGFR} upon TNF treatment (Figure 2B). Similar results were obtained in PC12 cell lines expressing the chimeric receptors TNFR2-TrkB (data not shown). These results show that activation of TrkA (or TrkB) tyrosine kinase is necessary and sufficient to induce the kinase activity associated with p75^{NGFR}.

Binding of NGF to p75^{NGFR} modulates the kinetics of p75^{NGFR}-associated protein kinase activation

As demonstrated above, the co-expression of p75^{NGFR} and TrkA is necessary for NGF-mediated p75^{NGFR}-associated

Fig. 3. Low NGF concentration results in rapid p75^{NGFR}-associated kinase activity. (A) Protein lysates from PC12 cells treated with 5 ng/ml NGF for 0, 2, 5 and 30 min were immunoprecipitated with MC192 antibody and the MBP kinase assay was performed. MBP phosphorylation was increased after 2 min of NGF treatment (lane 2). (B) PC12 cells were treated with 50 ng/ml of NGF for 0, 2, 5 and 30 min and samples processed as in (A). MBP phosphorylation was increased after 5 min NGF treatment (lane 3). (C) PC12 cells were treated with 5, 25 or 50 ng/ml of NGF for 0, 2, 3.5 and 5 min. Immunoprecipitation and MBP kinase assay were performed as in (A). Kinase activity was evaluated by counting the excised MBP bands. The error bars represent the variation of two experiments. Comparable p75^{NGFR} protein expression levels were used in each experiment.

kinase activation, suggesting that interaction between the NGF receptors may occur. One condition under which p75^{NGFR} and TrkA interaction is known to occur is at a limiting concentration of ligand. At low NGF concentrations, p75^{NGFR} functions to increase ligand binding of NGF to TrkA in PC12 cells, whereas, at higher NGF concentrations (50 ng/ml), this effect is lost (Barker and Shooter, 1994). To ascertain whether p75^{NGFR}-associated protein kinase activation depends on NGF concentration, PC12 cells were treated with either 5 or 50 ng/ml NGF. PC12 cells treated with 50 ng/ml NGF showed an ~4-fold increase in p75^{NGFR}-associated kinase activity with a peak activity at 5 min (Figure 3B and C); this activity subsequently declined to background levels by 10 min (data not shown). In contrast, at 5 ng/ml NGF, the kinase activation increased ~2-fold, but with peak activity at 2 min, followed by a decline to basal levels by 5 min (Figure 3A and C). An intermediate time course was seen at 25 ng/ml NGF with peak activity at 3.5 min (3- to 4-fold increase) (Figure 3C). Thus, at low NGF concentrations, the activation of the p75^{NGFR}-associated kinase is significantly accelerated. At either concentration, the p75^{NGFR}-associated protein kinase activity is transiently induced in PC12 cells upon NGF treatment. Although TrkA activation is necessary for p75^{NGFR}-associated kinase activity, the kinetics of TrkA activation are clearly different from those of the p75^{NGFR}-associated kinase. Peak TrkA tyrosine phosphorylation occurs at 5 min at both 5 and 50 ng/ml NGF (data not shown) and persists for 1 h.

Although NGF binding to p75^{NGFR} is not necessary for activation of its associated protein kinase, two independent experimental paradigms show that NGF binding to p75^{NGFR} is necessary for the accelerated activation of the kinase. The first used a mutated form of NGF (NGF^{3T}) that does not bind p75^{NGFR} but still activates TrkA (Ibáñez *et al.*, 1992). In contrast to wild-type NGF, treatment of PC12 cells with 5 and 50 ng/ml NGF^{3T} produced identical kinetics of p75^{NGFR}-associated protein kinase activation with a 5 min peak of activity (Figure 4A). Second, a 100-fold excess of brain-derived neurotrophic factor (BDNF)

Fig. 4. Accelerated activation requires NGF binding to p75^{NGFR}. (A) PC12 cells were treated with COS cell-derived NGF^{3T} at concentrations comparable with 5 and 50 ng/ml of wild-type NGF (NGF^{3T} concentrations were determined empirically by comparing the level of TrkA tyrosine phosphorylation with that produced by NGF). Kinase assay with MBP as substrate was performed on MC192 immunoprecipitates as before. Both 5 and 50 ng/ml NGF^{3T} demonstrated a peak MBP kinase activity at 5 min (lanes 3 and 5). (B) PC12 cells were treated with 5 ng/ml NGF for 0, 2 and 5 min in the presence or absence of 500 ng/ml BDNF. p75^{NGFR}-associated kinase activity was determined as before with MBP as substrate. Pre-treatment with 500 ng/ml BDNF for 5 min prior to NGF treatment shifts the peak of NGF-induced p75^{NGFR}-associated kinase activity from 2 to 5 min. The error bars demonstrate the variation of two separate experiments. Comparable p75^{NGFR} protein expression levels were used in each experiment.

was used in order to compete with NGF binding to p75^{NGFR} (Barker and Shooter, 1994). In the presence of 500 ng/ml BDNF, the peak activation of p75^{NGFR}-associated protein kinase produced by 5 ng/ml NGF was shifted from 2 to 5 min (Figure 4B). These levels of BDNF greatly decrease the magnitude of NGF-mediated TrkA tyrosine phosphorylation (Barker and Shooter, 1994) and account for the lower level of p75^{NGFR}-associated kinase activation observed in the presence of BDNF (Figure 4B). Together, these findings show that the accelerated activation of the p75^{NGFR}-associated protein kinase seen at low NGF concentrations requires NGF binding to p75^{NGFR}.

The p75^{NGFR}:TrkA ratio affects the p75^{NGFR}-associated protein kinase activation

In PC12 cells, the ratio of p75^{NGFR} to TrkA molecules has been estimated at 10–20:1 (Hempstead *et al.*, 1991; Battleman *et al.*, 1993). To investigate the effect of the p75^{NGFR}:TrkA ratio on the activation of the p75^{NGFR}-associated kinase, we generated PC12 cell lines stably expressing antisense cRNA for p75^{NGFR}. In one of these lines (OPαp75-5), the p75^{NGFR} protein level was reduced by ~90% compared with wild-type PC12 cells (Figure 5B); the levels of TrkA were unaffected (data not shown). As expected, the OPαp75-5 cells have reduced p75^{NGFR}-associated kinase activity in response to NGF compared with wild-type PC12 cells. Treatment with 5 ng/ml NGF produced a peak in p75^{NGFR}-associated protein kinase activity at 5 min rather than at 2 min (Figure 5A). Therefore, decreasing the ratio of p75^{NGFR} to TrkA from ~10–20:1 to 1–2:1 prevented the rapid activation of p75^{NGFR}-associated protein kinase normally observed at low NGF concentrations.

Fig. 5. Reduction of the p75^{NGFR}:TrkA ratio prevents rapid kinase activation. (A) OP α p75-5 cells, a PC12 cell line expressing antisense p75^{NGFR} cRNA resulting in reduced p75^{NGFR} protein levels, were treated with 5 ng/ml NGF for 0, 2, 5 and 30 min (lanes 5–8). Wild-type PC12 cells were similarly treated (lanes 1–4). p75^{NGFR}-associated kinase activity was determined as before with MBP as substrate. The PC12 cells showed peak kinase activity after 2 min (lane 2). With the decreased p75^{NGFR}:TrkA ratio in the OP α p75-5 cells, maximum MBP phosphorylation occurred at 5 min, despite the low NGF concentration (lane 7). (B) Western blot analysis of MC192 immunoprecipitates from PC12 cells and OP α p75-5 cells (lanes 1 and 2, respectively). p75^{NGFR} expression was reduced by ~90% in the OP α p75-5 cells as determined by densitometric scanning.

Fig. 6. Characterization of p75^{NGFR}-associated protein kinase in dissociated DRG cells in culture. (A) Dissociated DRG cells in culture were treated with NGF (50 ng/ml for 5 min) and p75^{NGFR} immunoprecipitated with MC192 antibody. Samples were electrophoresed on 8% SDS-PAGE containing 0.5 mg/ml MBP, and the kinase reaction was performed in the gel. A protein of 120 kDa displays kinase activity. (B) Western blot analysis of aliquots of each immunoprecipitate from dissociated DRG samples showed comparable levels of p75^{NGFR} protein (lanes 1 and 2 respectively).

Characterization of the p75^{NGFR}-associated protein kinase in DRG and PC12 cells

As an initial step towards identifying the p75^{NGFR}-associated protein kinase, we used an in gel renaturation protein kinase assay in which proteins are renatured in a gel impregnated with MBP. By this method, we identified MBP kinase activity in a DRG cell lysate prepared after NGF (50 ng/ml) treatment in a band of ~120 kDa that co-immunoprecipitates with p75^{NGFR} (Figure 6A, lane 2). In agreement, in PC12 cells we have identified MBP kinase activity in bands of ~120, 56 and 44 kDa that co-

Fig. 7. A 120/104 kDa protein kinase is rapidly activated at low NGF concentrations. (A) MC192 immunoprecipitates from protein lysates (2 mg) of PC12 cells treated with 5 or 50 ng/ml for 0, 2 and 5 min. Samples were electrophoresed on 8% SDS-PAGE containing 0.5 mg/ml MBP, and the kinase reaction was performed in the gel. Proteins of 120, 56 and 44 kDa display kinase activity. (B) Immunoprecipitates using 0.3 mg of protein lysate from PC12 cells treated as in (A) were separated on 12% SDS-PAGE containing 0.5 mg/ml MBP. The in gel kinase assay was performed, revealing that the upper band (A) migrated as a doublet of 120/104 kDa with rapid kinase activation at low NGF concentrations. The lower molecular weight proteins were not detectable at this protein concentration.

immunoprecipitate with p75^{NGFR} (Figure 7A). The 120 kDa protein was resolved further as a doublet of 120 and 104 kDa when the amount of protein lysate used for immunoprecipitation was decreased from 2 to 0.3 mg (Figure 7B). Under the conditions of reduced lysate protein, no NGF-independent basal level of MBP phosphorylation was observed, demonstrating the specificity of the interaction of the 120/104 kDa kinase with p75^{NGFR}.

Several lines of evidence indicate that the 120/104 kDa protein represents the predominant p75^{NGFR}-associated protein kinase activity. First, the 120/104 kDa protein that co-immunoprecipitates with p75^{NGFR} represented the major source of MBP protein kinase activity in PC12 and DRG cells. Gel kinase assays performed without MBP showed minimally detectable autophosphorylation of the 120/104 kDa protein in response to NGF (data not shown). Furthermore, in PC12 cells, the activation of the 120/104 kDa protein kinase was dependent upon NGF concentration. In parallel experiments, 5 ng/ml NGF treatment resulted in rapid activation of the 120/104 kDa protein kinase with a peak activity at 2 min and a return to basal levels by 5 min (Figure 7B, lanes 2 and 3). In contrast, 50 ng/ml NGF caused minimal activation of the 120/104 kDa protein kinase at 2 min and maximal activation at 5 min (Figure 7B, lanes 4 and 5).

The kinase activity detected at ~44 kDa probably represents ERK 1 and/or 2, as previously reported (Volonté *et al.*, 1993b), and it is possible that the 56 kDa protein may represent ERK 3 (Boulton *et al.*, 1991). The 44 and 56 kDa kinases did not show accelerated activation at low NGF concentration (Figure 7A, lane 2), but did display elevated levels of MBP phosphorylation after 2 and 5 min of 50 ng/ml NGF treatment (Figure 7A, lanes 2–6). Despite detection of the 42 and 44 kDa proteins with polyclonal anti-ERK antibody in immunoblots of p75^{NGFR} immunoprecipitates, we have been unable to identify tyrosine phosphorylated proteins in this molecular range in p75^{NGFR} immunoprecipitates using anti-phosphotyrosine antibodies (data not shown). Thus, in both PC12 and DRG cells, the majority of the MBP-directed kinase activity associated with p75^{NGFR} is associated with the 120/104 kDa proteins.

Fig. 8. p75^{NGFR} deletion mutants and associated kinase activity. (A) p75^{NGFR} receptor and the two p75^{NGFR} mutants carrying a deletion in the last 82 (p75^{#1}) or 38 (p75^{#2}) amino acid residues respectively are schematically represented in the upper panel. The black box represents the extracellular domains and the white box the cytoplasmic domains; arrows indicate the number of amino acid residues that comprise each receptor (395 for p75^{NGFR} wild-type, and 313 or 357 for p75^{#1} or p75^{#2} mutants respectively). The lower panel shows receptor protein expression levels detected by Western blot after transient transfection in COS7 cells (lane 1, p75^{NGFR}; lane 2, p75^{#1}; lane 3, p75^{#2}). (B) 3T3-TrkA cells were transfected with p75^{NGFR}, p75^{#1} or p75^{#2} and treated with serum-free medium (black columns) or NGF 50 ng/ml (cross-hatched columns) for 5 min. The receptors were immunoprecipitated from cell lysates using a monoclonal antibody (MC192) that recognizes their extracellular domains. A cell-free protein kinase assay was performed by incubating the immunocomplexes with MBP as substrate. NGF treatment of 3T3-TrkA cells expressing p75^{NGFR} and p75^{#2} enhanced their associated kinase activity as evaluated by counting the excised MBP bands. No NGF-dependent activity was associated with the p75^{#1} mutant. The error bars represent the variation of three experiments. The levels of expression of the wild-type and mutant p75^{NGFR}s were the same in the NIH-3T3 cells as shown for the COS7 cells. (C) 3T3-TrkA cells were transfected with p75^{NGFR}, p75^{#1} or p75^{#2}, treated with serum-free medium or NGF 50 ng/ml for 5 min and the receptors immunoprecipitated with MC192 antibody. Samples were electrophoresed on 8% SDS-PAGE containing 0.5 mg/ml MBP, and the kinase reaction was performed in the gel. Proteins of 120 and 104 kDa showed NGF-induced kinase activity associated with the p75^{NGFR} and p75^{#2} receptors, whereas p75^{#1} did not show NGF-dependent MBP phosphorylation.

Identification of p75^{NGFR} cytoplasmic region required for the 120/104 kDa protein kinase activity

To identify initially the regions of p75^{NGFR} that either directly or indirectly functionally associate with the 120/104 kDa protein kinase, deletion mutants of the cytoplasmic domain of p75^{NGFR} were designed. Two mutants carrying a deletion of 82 (p75^{#1}) and 38 (p75^{#2}) amino acid residues of the carboxyl-terminal region of p75^{NGFR} were constructed and transfected in COS7 cells to monitor protein expression (Figure 8A). A two-receptor system was then reconstituted in NIH-3T3 cells stably expressing TrkA receptors (3T3-TrkA) by transient transfection with p75^{NGFR}, p75^{#1} or p75^{#2} receptors. Protein kinase activity that co-immunoprecipitated with p75^{NGFR}, p75^{#1} or p75^{#2} mutants was observed in a cell-free phosphorylation assay using MBP as substrate (Figure 8B) and by an in gel renaturation protein kinase assay (Figure 8C). NGF-

dependent kinase activity associated with p75^{NGFR} and p75^{#2} receptors was detected in both 120 and 104 kDa proteins. On the contrary, no significant increased kinase activity was associated with the p75^{#1} mutant. NGF treatment of 3T3-TrkA cells transfected with vector alone showed only background kinase activity in MC192 immunocomplexes (data not shown). Although the p75^{NGFR} protein expression levels for p75^{#1} were less than for the other mutant and wild-type p75^{NGFR}, this would not affect the kinase activation results because p75^{NGFR} levels can be varied several fold without affecting the activation of the kinase (Figures 5 and 7). These results show that a functional interaction between p75^{NGFR} and the 120/104 kDa protein kinase is retained in the presence of a 43 amino acid region between Ser314 and Thr357 of the p75^{NGFR} cytoplasmic domain.

Discussion

The role of p75^{NGFR} in NGF signal transduction has been the subject of much debate (Chao, 1992; Meakin and Shooter, 1992; Barbacid, 1993; Chao and Hempstead, 1995). To address a possible functional role for p75^{NGFR} in NGF signal transduction, we have investigated how NGF activates a protein kinase associated with p75^{NGFR}. Our results provide two new pieces of evidence that p75^{NGFR} participates in NGF signaling. First, we have detected a previously undescribed 120/104 kDa protein kinase that is associated with p75^{NGFR}. Second, we found that TrkA and p75^{NGFR} collaborate to activate this p75^{NGFR}-associated kinase. At present, we cannot distinguish whether the 120/104 kDa kinase is constitutively associated with p75^{NGFR} or is recruited upon NGF treatment.

p75^{NGFR} is associated with a 120/104 kDa protein kinase

A 120/104 kDa protein kinase associated with p75^{NGFR} was activated in DRG, PC12 and 3T3-TrkA cells upon NGF treatment. This protein kinase represents the majority of the NGF-dependent kinase associated with p75^{NGFR} although, in PC12 cells, two other protein kinases of ~44 and 56 kDa respectively were detected. The 120/104 kDa protein kinase is not likely to be the same as those previously shown to be associated with p75^{NGFR}; ERKs 1 and 2 and as yet unidentified phosphoproteins of 60 and 130 kDa (Omichi *et al.*, 1991; Volonté *et al.*, 1993b). We were able to co-immunoprecipitate only very low levels of ERKs 1 and 2 with p75^{NGFR}. In contrast, the vast majority of p75^{NGFR}-associated kinase was seen in the 120/104 kDa proteins. Moreover, the 120/104 kDa kinase doublet was detectable by immunoprecipitation from as little as 300 µg of protein lysate, conditions under which we could not detect ERKs by the in gel kinase assay. The rapid activation of the 120/104 kDa protein kinase suggests that it lies upstream, or is independent, of ERKs in the NGF signal transduction cascade. Though the 120/104 kDa kinase we have identified is of similar size to the 130 kDa phosphoprotein reported by Ohmichi *et al.* (1991), no kinase activity was previously ascribed to this p75^{NGFR}-associated protein.

NGF stimulation of p75^{NGFR} induces the accumulation of ceramide (Dobrowsky *et al.*, 1994). In HL60 cells, a 97 kDa ceramide-activated kinase has been characterized

that phosphorylates MBP and is autophosphorylated on serine residues (Liu *et al.*, 1994). These results raise the possibility of whether ceramide generation upon NGF binding to p75^{NGFR} may lead to accelerated activation of the p75^{NGFR}-associated kinase. Since we observed no activation of the p75^{NGFR}-associated kinase in the absence of TrkA phosphorylation and no protein kinase activity associated with p75^{NGFR} in PC12 cells treated with a synthetic homolog of ceramide (data not shown), its activation is unlikely to be purely ceramide dependent. Thus, the p75^{NGFR}-associated 120/104 kDa protein kinase is most likely not the 97 kDa ceramide-activated kinase described by Liu *et al.* (1994).

The region of functional interaction between p75^{NGFR} and 120/104 kDa protein kinase was investigated by measuring the MBP kinase activity associated with p75^{NGFR} mutants. The deletion analysis of the p75^{NGFR} cytoplasmic domain demonstrated that 120/104 kDa protein kinase activity is only retained if a 43 amino acid region between Ser314 and Thr357 is conserved; thus, this region is required for the activation and/or association of the p75^{NGFR}-associated protein kinase. This region does not correspond to the putative 'death domain' of p75^{NGFR}, an essential domain, initially identified in TNFR1 and TNFR2 receptors of the same family, responsible for the protein-protein interaction involved in the transduction of apoptotic signals (Boldin *et al.*, 1995; Feinstein *et al.*, 1995). Furthermore, deletion of the 'death domain' of p75^{NGFR} (p75^{#2}) did not prevent the NGF-dependent 120/104 kDa protein kinase activity.

p75^{NGFR} and TrkA collaborate to activate the 120/104 kDa p75^{NGFR}-associated protein kinase

Neurotrophins all share the ability to bind to p75^{NGFR} as well as to their cognate Trk receptors. Although Trk receptors are required and sufficient to promote neurite outgrowth and cell survival, several lines of evidence support the notion that these receptors collaborate to transduce NGF-mediated signaling (Chao and Hempstead, 1995). In support of this idea, we now demonstrate that the activation of a 120/104 kDa protein kinase is accelerated at low compared with high NGF concentrations in a process that requires both TrkA tyrosine phosphorylation and NGF binding to p75^{NGFR}. The p75^{NGFR}-associated kinase is not directly activated by NGF binding to p75^{NGFR}, as shown by the failure of NGF to activate the kinase in a variant PC12 cell line lacking TrkA. On the other hand, phosphorylation of TrkA alone using the mutated NGF^{3T} that does not bind to p75^{NGFR}, or phosphorylation of the chimeric TNFR2-TrkA receptor by TNF that also does not bind to p75^{NGFR}, is sufficient for the activation of the kinase. However, the accelerated activation of the p75^{NGFR} kinase is blocked if NGF binding to p75^{NGFR} is inhibited by BDNF or if the ratio of p75^{NGFR} to TrkA is reduced ~10-fold. This requirement for NGF binding to both p75^{NGFR} and TrkA for rapid kinase activation suggests a functional cytoplasmic coupling of these receptors. Such a coupling could be affected by direct phosphorylation of 120/104 kDa kinase by TrkA. We have so far, however, failed to detect tyrosine phosphorylation of this kinase in p75^{NGFR} immunoprecipitates, implying an intermediate step in its activation (data not shown; Omichi *et al.*, 1991; Volonté *et al.*, 1993b). It was

noted previously that a partial deletion of the cytoplasmic domain of the p75^{NGFR} results in loss of NGF signaling in PC12 cells and gain of TrkA function in 3T3 cells (Hempstead *et al.*, 1990; Berg *et al.*, 1991; Hantzopoulos *et al.*, 1994). Furthermore, it has been demonstrated recently that Trk modulates p75^{NGFR}-dependent sphingomyelin hydrolysis in PC12 cells, suggesting that cross-talk pathways occur between p75^{NGFR} and Trk-dependent cell signaling (Dobrowsky *et al.*, 1995). Whether the p75^{NGFR}-associated kinase is responsible for these effects is not known, but it is clear that the characterization of this kinase and the determination of its localization will provide a more thorough understanding of p75^{NGFR}-TrkA interactions and of the immediate events in NGF signal transduction.

Materials and methods

Cell culture

COS7 and PC12 cells were cultured in DMEM supplemented with 6% bovine calf serum, 6% horse serum at 37°C and 5% or 12% CO₂ respectively. 3T3-TrkA cells were cultured in 10% bovine calf serum and 2.5 mM histidinol. Primary DRG cells were cultured in DMEM with 10% fetal calf serum enriched with NGF (100 ng/ml) at 37°C and 7% CO₂. For treatment with trophic factors, DMEM supplemented with 0.1% bovine serum albumin was used.

Preparation of dissociated DRG

Post-natal day 9 rat DRG were dissociated with 0.25% trypsin in L15 medium for 1 h at 37°C. Non-neuronal cells were partially removed by pre-plating the dissociated DRG for 2 h in a 10 cm plastic dish at 37°C and 7% CO₂. The remaining dissociated DRG were plated in a collagen-coated 10 cm dish and cultured for 2 days in the presence of NGF 100 ng/ml enriched medium.

Production of transfected cell lines

For cell lines expressing antisense p75^{NGFR}, PC12 cells were transfected with pOPαp75 [i.e. 5' 756 bp of rat p75^{NGFR} cDNA (Radeke *et al.*, 1987) cloned into *NotI* sites of pOP13CAT (Stratagene) in reverse orientation] using LipofectAMINE (Gibco-BRL). Stable lines were selected in 400 µg/ml geneticin and maintained in 100 µg/ml geneticin (Gibco-BRL). cRNA expression and protein suppression were verified by Northern and Western blots, respectively. Production of the pcTA, pcTK, pcTL chimeric receptor cell lines has been described previously (Rovelli *et al.*, 1993; Canossa *et al.*, 1994). 3T3-TrkA cells were provided by Dr George Yancopoulos.

Ligands and antibodies

Mouse NGF was purchased from Harland Bioproducts for Science. Recombinant human BDNF was a gift from G.D.Yancopoulos. COS cell-derived mouse NGF^{3T} was a gift from P.A.Barker (Barker and Shooter, 1994). Human TNF and monoclonal antibody to the extracellular domain of the human TNFR2 receptor, utr-4, were gifts from Drs W.Lesslauer and H.Loetscher. Additional antibodies used included: anti-rat p75^{NGFR} mouse monoclonal ascites (MC192; Chandler *et al.*, 1984) and rabbit polyclonal antiserum generated against p75^{NGFR} intracellular domain fusion protein (a gift from Dr S.J.Decker; Omichi *et al.*, 1991) or a peptide from the cytoplasmic domain of p75^{NGFR}, αp75-p1 (Barker *et al.*, 1994).

Immunoprecipitation

Dissociated DRG in culture and PC12 cells were washed twice with DMEM and serum starved for 1 h before NGF treatment, lysed in 500 µl of lysis buffer (20 mM Tris-Cl pH 7.4, 150 mM NaCl, 1% Triton X-100, 20 mM NaF, 2 mM *n*-nitrophenyl phosphate, 1 µg/ml leupeptin and 2 mM sodium orthovanadate; Volonté *et al.*, 1993a) and protein concentration determined by Bradford assay (Bio-Rad). Lysates were equalized for protein content and used directly for immunoprecipitation at 4°C overnight with 5 µl of MC192 ascites fluid or 5 µl of polyclonal anti-p75^{NGFR} antiserum (Omichi *et al.*, 1991). Immunocomplexes were pelleted with protein A/G⁺ agarose (Santa Cruz Biologicals) or anti-mouse IgG agarose (Sigma) at 4°C for 1–2 h. Complexes were washed

with lysis buffer three times and then processed for Western blots or *in vitro* kinase assays.

Western blotting

p75^{NGFR} immunoprecipitates were boiled for 5 min in Laemmli sample buffer (LSB), subjected to SDS-PAGE and electroblot transferred to nitrocellulose. Non-specific protein binding was reduced by pre-incubating blots in 5% non-fat dry milk in 10 mM Tris-Cl (pH 7.4), 150 mM NaCl and 0.04% Tween-20 (TBS). Blots were incubated with a 1:1000 dilution of the primary antibody, α p75-p1, in TBS with 5% non-fat dry milk overnight at 4°C. Blots were washed extensively in TBS, incubated with protein A-horseradish peroxidase diluted 1:1500 as above for 1 h at room temperature, and then washed in TBS. Immunoblots were developed using the ECL detection system (Amersham).

Kinase assays

p75^{NGFR} immunocomplexes were washed in kinase buffer (10 mM Tris-Cl pH 7.4, 150 mM NaCl, 10 mM MgCl₂, and 0.5 mM dithiothreitol) twice and then incubated for 15 min at 37°C in 40 μ l of kinase buffer containing 25 mM ATP, 2.5 μ Ci [γ -³²P]ATP and 1.0 mg/ml MBP. Reactions were stopped by boiling in LSB and samples subjected to SDS-PAGE. The gels were fixed, Coomassie stained, dried and exposed to X-ray film. MBP phosphorylation was quantified by Cerenkov counting of MBP bands excised from the gel. For *in gel* renaturation kinase assays, MC192 immunocomplexes were separated on SDS-polyacrylamide gels which were polymerized in the presence of 0.5 or 1 mg/ml MBP and the kinase assay performed as reported by Campos-González and Glenney (1992). Gels without impregnated exogenous substrate were processed similarly to investigate possible autophosphorylation of proteins co-immunoprecipitating with p75^{NGFR}. All *in vitro* kinase assays were repeated independently at least three times.

Construction of p75^{NGFR} deletion mutants

Using the p75^{NGFR} receptor, subcloned in pCDM8 expression vector (Invitrogen), as template, a one-step PCR strategy was employed to delete the last 82 (p75^{#1}) or 38 (p75^{#2}) amino acid residues of the carboxy-terminal tail. The PCRs were performed using a specific 5' sense oligonucleotide including the initiation codon (ATG), of p75^{NGFR} (5'-GCTCTAGAGCATGAGGAGGGCAGGTGC-3') and 3' antisense oligonucleotides with a translation stop codon (TCA) inserted (5'-GCTCTAGATCAGGTATCCCCGTTGAGCA-3' for the p75^{#1} construct and 5'-GCTCTAGATCACTGGCTGTCCACAGAGA-3' for the p75^{#2} construct). The resulting cDNA for the constructs p75^{#1} and p75^{#2} were subcloned into pCDM8 and sequenced.

Transfection of p75^{NGFR} deletion mutants in COS7 and 3T3-TrkA cell lines

Transfection of the p75^{NGFR}, p75^{#1} and p75^{#2} in COS7 cells was carried out by the DEAE dextran procedure (Gorman, 1985). The day before the transfection, 1 $\times$ 10⁶ cells were plated into 10 cm dishes; 48 h after the transfection cells were washed once and incubated for 1 h at 37°C with serum-free DMEM. Lysed cells, solubilized in a volume of 500 μ l of RIPA buffer (10 mM Tris pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% deoxycholic acid, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride, 0.1 mM benzamide, 0.05 mM 1,10-phenanthroline protease inhibitor), were immunoprecipitated and protein expression levels detected by Western blot as above. NIH-3T3 fibroblasts expressing TrkA receptors (3T3-TrkA) were transiently transfected, using the LipofectAMINE (GIBCO-BRL) strategy, with 6 μ g of an expression vector coding for p75^{NGFR}, p75^{#1} and p75^{#2} receptors. After NGF treatment, the receptors were immunoprecipitated from the cell lysate and either the '*in vitro*' MBP phosphorylation or '*in gel*' kinase assay performed.

Acknowledgements

The authors are grateful to Barbara Barres, Howard Schulman, Philip Barker, John Heymach and Carlo Jannicola for proofreading, and to members of the Shooter Laboratory who provided useful comments on this work. George Yancopoulos provided recombinant BDNF and NIH-3T3 cells expressing TrkA; Philip Barker provided mutated mouse NGF and polyclonal anti-p75^{NGFR} antibody; Stuart Decker provided antibody to the cytoplasmic domain of p75^{NGFR}; Giorgio Rovelli provided pcTL cells; and W.Lesslauer and H.Loetscher provided human TNF and anti-TNFR2 antibody. This work was supported by grants from NINDS/NIH (K08-NS01706 to J.L.T. and NS04270 to E.M.S.) and the Alzheimer's Association. M.C. was supported by fellowships from Società Italiana

di Farmacologia and Centro Interdipartimentale di Recherche Biotecnologiche and A.N.V. by Training grant 2T32HDO749.

References

- Barbacid, M. (1993) The Trk family of neurotrophin receptors: molecular characterization and oncogenic activation in human tumors. In Levine, A.J. and Schimdek, H.H. (eds), *Molecular Genetics of Nervous System Tumors*. John Wiley & Sons, Inc., New York, pp. 123–135.
- Barker, P.A. and Shooter, E.M. (1994) Disruption of NGF binding to the low affinity neurotrophin receptor p75^{LNT} reduces NGF binding to TrkA on PC12 cells. *Neuron*, **13**, 203–215.
- Barker, P.A., Barbee, G., Misko, T.P. and Shooter, E.M. (1994) The low affinity neurotrophin receptor, p75^{LNT}, is palmitoylated by thioester formation through cysteine 279. *J. Biol. Chem.*, **269**, 30645–30650.
- Barrett, G.L. and Bartlett, P.F. (1994) The p75 nerve growth factor receptor mediates survival or death depending on the stage of sensory neuron development. *Proc. Natl Acad. Sci. USA*, **91**, 6501–6505.
- Battleman, D.S., Geller, A.I. and Chao, M.V. (1993) HSV-1 vector-mediated transfer of the human nerve growth factor receptor p75hNGFR defines high-affinity NGF binding. *J. Neurosci.*, **13**, 941–951.
- Benedetti, M., Levi, A. and Chao, M.V. (1993) Differential expression of nerve growth factor receptors leads to altered binding affinity and neurotrophin responsiveness. *Proc. Natl Acad. Sci. USA*, **90**, 7859–7863.
- Berg, M.M., Sternberg, D.W., Hempstead, B.L. and Chao, M.V. (1991) The low affinity p75 nerve growth factor (NGF) receptor mediates NGF-induced tyrosine phosphorylation. *Proc. Natl Acad. Sci. USA*, **88**, 7106–7110.
- Boldin, M.P., Varfolomeev, E.E., Pancer, Z., Mett, I.L., Camonis, J.H. and Wallach, D. (1995) A novel protein that interacts with the death domain of Fas/APO1 contains a sequence motif related to the death domain. *J. Biol. Chem.*, **270**, 7795–7798.
- Boulton, T.G., Nye, S.H., Robbins, D.J., Ip, N.Y., Radziejewska, E., Morgenbesser, S.D., DePinho, R.A., Panayotatos, N., Cobb, M.H. and Yancopoulos, G.D. (1991) ERKs: a family of protein-serine/threonine kinases that are activated and tyrosine phosphorylated in response to insulin and NGF. *Cell*, **65**, 663–675.
- Campos-Gonzalez, R. and Glenney, J.R., Jr (1992) Tyrosine phosphorylation of mitogen-activated protein kinase in cells with tyrosine kinase-negative epidermal growth factor receptors. *J. Biol. Chem.*, **267**, 14535–14538.
- Canossa, M., Verity, A.N., Rovelli, G. and Shooter, E.M. (1994) Chimeric TNFR2–Trk receptors: a tool to investigate Trk-mediated gene expression. *Soc. Neurosci. Abstr.*, **20**, 38.
- Chandler, C.E., Parsons, L.M., Hosang, M. and Shooter, E.M. (1984) A monoclonal antibody modulates the interaction of nerve growth factor with PC12 cells. *J. Biol. Chem.*, **259**, 6882–6889.
- Chao, M.V. (1992) Neurotrophins receptors: a window into neuronal differentiation. *Neuron*, **9**, 583–593.
- Chao, M.V. and Hempstead, B.L. (1995) p75 and TrkA, a two receptor system. *Trends Neurosci.*, **18**, 321–326.
- Dobrowsky, R.T., Werner, M.H., Castellino, A.M., Chao, M.V. and Hannun, Y.A. (1994) Activation of the sphingomyelin cycle through the low-affinity neurotrophin receptor. *Science*, **265**, 1596–1599.
- Dobrowsky, R.T., Jenkins, G.M. and Hannun, Y.A. (1995) Neurotrophins induce sphingomyelin hydrolysis. *J. Biol. Chem.*, **270**, 22135–22142.
- Feinstein, E., Wallach, D., Boldin, M.P., Varfolomeev, E.E. and Kimchi, A. (1995) The death domain: a module shared by proteins with diverse cellular functions. *Trends Biochem. Sci.*, **20**, 342–344.
- Gorman, C. (1985) *DNA Cloning: A Practical Approach*. Vol. 2. IRL Press, Oxford, UK.
- Green, S.H., Rydel, R.E., Connolly, J.L. and Greene, L.A. (1986) PC12 cell mutants that possess low but not high affinity nerve growth factor receptors neither respond to nor internalize nerve growth factor. *J. Cell Biol.*, **102**, 830–843.
- Hantzopoulos, P.A., Suri, C., Glass, D.J., Goldfarb, M.P. and Yancopoulos, G.D. (1994) The low affinity NGF receptor, p75, can collaborate with each of the Trks to potentiate functional responses to the neurotrophins. *Neuron*, **13**, 187–201.
- Hempstead, B.L., Patil, N., Thiel, B. and Chao, M.V. (1990) Deletion of cytoplasmic sequences of the nerve growth factor receptor leads to loss of high affinity ligand binding. *J. Biol. Chem.*, **265**, 9595–9598.

- Hempstead,B.L., Martin-Zanca,D., Kaplan,D.R., Parada,L.F. and Chao,M.V. (1991) High-affinity NGF binding requires coexpression of the *trk* proto-oncogene and the low-affinity NGF receptor. *Nature*, **350**, 678–683.
- Ibáñez,C.F., Ebendal,T., Barbany,G., Murray-Rust,J., Blundell,T.L. and Persson,H. (1992) Disruption of the low affinity receptor-binding site in NGF allows neuronal survival and differentiation by binding to the *trk* gene product. *Cell*, **69**, 329–341.
- Kaplan,D., Hempstead,B., Martin-Zanca,D., Chao,M. and Parada,L. (1991) The *trk* proto-oncogene product: a signal transducing receptor for nerve growth factor. *Science*, **252**, 554–558.
- Knipper,M., Beck,A., Rylett,J. and Breer,H. (1993) Neurotrophin induced cAMP and IP₃ responses in PC12 cells. *FEBS Lett.*, **324**, 147–152.
- Liu,J., Mathias,S., Yang,Z. and Kolesnick,R.N. (1994) Renaturation and tumor necrosis factor- α stimulation of a 97-kDa ceramide-activated protein kinase. *J. Biol. Chem.*, **269**, 3047–3052.
- Mahedeo,D., Kaplan,L., Chao,M.V. and Hempstead,B.L. (1994) High affinity nerve growth factor binding displays a faster rate of association than p140^{trk} binding. *J. Biol. Chem.*, **269**, 6884–6891.
- Meakin,S.O. and Shooter,E.M. (1992) The neurotrophin receptors. *Trends Neurosci.*, **15**, 323–331.
- Omichi,M., Decker,S.J. and Saltiel,A.R. (1991) Nerve growth factor induces the association of a 130 kDa phosphoprotein with its receptor in PC-12 pheochromocytoma cells. *Cell Regul.*, **2**, 691–697.
- Rabizadeh,S., Oh,J., Zhong,L.T., Yang,J., Bitler,C.M., Butcher,L.L. and Bredesen,D.E. (1993) Induction of apoptosis by the low-affinity NGF receptor. *Science*, **261**, 345–348.
- Radeke,M.J., Misko,T.P., Hsu,C., Herzenberg,L.A. and Shooter,E.M. (1987) Gene transfer and molecular cloning of the rat nerve growth factor receptor. *Nature*, **325**, 593–597.
- Rovelli,G., Heller,R.A., Canossa,M. and Shooter,E.M. (1993) Chimeric tumor necrosis factor–TrkA receptors reveal that ligand-dependent activation of the TrkA tyrosine kinase is sufficient for differentiation and survival of PC12 cells. *Proc. Natl Acad. Sci. USA*, **90**, 8717–8721.
- Snider,W.D. (1994) Function of the neurotrophins during nervous system development: what the knockouts are teaching us. *Cell*, **77**, 627–638.
- Verdi,J.M., Birren,S.J., Ibáñez,C.F., Persson,H., Kaplan,D.R., Benedetti,M., Chao,M.V. and Anderson,D.J. (1994) p75^{LNGFR} regulates Trk signal transduction and NGF-induced neuronal differentiation in MAH cells. *Neuron*, **12**, 733–745.
- Volonté,C., Ross,A.H. and Greene,L.A. (1993a) Association of a purine-analogue-sensitive protein kinase activity with p75 nerve growth factor receptors. *Mol. Biol. Cell*, **4**, 71–78.
- Volonté,C., Angelastro,J.M. and Greene,L.A. (1993b) Association of protein kinases ERK1 and ERK2 with p75 nerve growth factor receptors. *J. Biol. Chem.*, **268**, 21410–21415.

Received on December 8, 1995; revised on February 14, 1996