THE JOURNAL OF BIOLOGICAL CHEMISTRY
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Vol. 272, No. 26, Issue of June 27, pp. 16322–16328, 1997
Printed in U.S.A.

Differential Modulation of Neuron Survival during Development by Nerve Growth Factor Binding to the p75 Neurotrophin Receptor*

(Received for publication, December 18, 1996, and in revised form, April 23, 1997)

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Nerve growth factor (NGF) supports the survival and differentiation of distinct populations of peripheral and central neurons. NGF binds to two classes of cell-surface receptors, the protein tyrosine kinase TrkA and the smaller p75 receptor lacking intrinsic catalytic activity. It has been suggested that both receptors are required for NGF high affinity binding, although TrkA appears to be sufficient for transducing most of the biological effects of NGF. Some evidence suggests that p75 could play a modulatory role on TrkA activation by an as yet unknown mechanism. In this study, we have investigated functional roles of p75 using a purified triple mutant NGF (triNGF) deficient in p75 binding but retaining significant TrkA binding and activation. The mutant was found to be as potent as wild type NGF at promoting survival of serum-deprived TrkA-expressing fibroblasts. On developing chick sensory neurons, survival responses to mutant and native NGF were indistinguishable when assayed at nanomolar concentrations. However, triNGF was 3- to 4-fold less potent than wild type NGF at lower concentrations (*i.e.* 10^{-11} M). Interestingly, in PC12 cells coexpressing TrkA and p75, no high affinity binding sites for triNGF could be detected. The reduced responsiveness to triNGF in sensory neurons was increasingly evident at later developmental stages; late embryonic neurons did not respond at all to concentrations of triNGF that were saturating at earlier developmental stages. Likewise, although no difference could be seen between wild type and mutant NGF on the survival responses of embryonic rat superior cervical ganglion sympathetic neurons, the mutant was much less potent than native NGF on postnatal sympathetic neurons. In sensory neurons, the decrease in responsiveness to triNGF correlated with a developmental reduction in the expression of both p75 and TrkA. Thus, NGF binding to p75 enhances responsiveness to ligand, particularly when this is present at limiting concentrations. During development, p75 modulates responsiveness to NGF so that binding to p75 becomes increasingly important in neurons undergoing a down-regulation of NGF receptors. These results support a ligand-dependent modulatory role for p75 in NGF-mediated neuron survival consistent with p75 functioning as a TrkA regulator and/or signaling receptor.

Neurotrophins are structurally and functionally related trophic factors involved in the development, survival, and maintenance of vertebrate neurons. In mammals four members have been characterized to date, nerve growth factor (NGF),[1] brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4 (NT-4) (1). Each neurotrophin supports distinct neuronal populations in the central and peripheral nervous systems. The main targets of NGF are sympathetic neurons as well as subpopulations of sensory and central neurons.

On NGF-responsive neurons, ^{125}I-NGF binding studies have shown two distinct classes of binding sites that differ in their affinity, a major class with a K_d of 10^{-9} M and a less abundant class with a K_d of 10^{-11} M (2, 3). The NGF association for both sites is very fast, being close to diffusion limited. However, the rate of dissociation differs markedly, being fast for the low affinity and slow for the high affinity site (4). The dose-response curves for neurotrophin-induced effects on neurons together with the finding that NGF expression *in vivo* is in the picomolar range have suggested that most biological effects are mediated by high affinity receptors (5, 6).

Intensive research eventually led to the identification of genes encoding two structurally unrelated classes of cell-surface receptors interacting with the neurotrophins, the common neurotrophin receptor p75 and members of the protein tyrosine kinase receptor family Trks. p75 is a transmembrane glycoprotein distantly related to the tumor necrosis factor receptor, CD40 and Fas/APO-1, and contains a short cytoplasmic tail lacking any known catalytic activity. p75 binds to all neurotrophins with equal affinity although with different rate constants (2, 7). Cells expressing only p75 display a single NGF-binding site with a K_d of $\approx 10^{-9}$ M and fast association and dissociation kinetics. p75 therefore exhibits the characteristics of the fast binding site found in NGF-responsive cells. Trks are single membrane spanning receptors with a large cytoplasmic domain that is autophosphorylated on tyrosine residues upon ligand binding. Binding and activation of different Trk receptors by neurotrophins is ligand-specific, *i.e.* NGF activates TrkA, BDNF and neurotrophin-4 activate TrkB, and neurotrophin-3 is the preferred ligand of TrkC. TrkA displays some of the properties of the high affinity NGF-binding site, most notably a slow dissociation of NGF (8). However, TrkA also shows binding characteristics that are not consistent with the kinetics of the slow site seen in neurons. The association rate of NGF to TrkA is slow, resulting in a K_d of $\approx 10^{-9}$ M. Several models have been proposed to account for the molecular composition of the

* This work was supported by grants from the Swedish Medical Research Council (to C. F. I.), by Public Health Service Grant NS 3081, and the Hirschl/Weill/Caulier Trust (to B. L. H.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: NGF, nerve growth factor; triNGF, triple mutant nerve growth factor; BDNF, brain-derived neurotrophic factor; NT-, neurotrophin-; wt, wild type; PAGE, polyacrylamide gel electrophoresis; DRG, dorsal root ganglion; SCG, superior cervical ganglion.

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high affinity binding site. Hempstead *et al.* (9) and Kaplan *et al.* (10) have postulated that NGF binds to either TrkA or p75 with low affinity, whereas co-expression of both receptors results in formation of high affinity binding sites. In contrast, equilibrium binding analyses demonstrated that a small number of high affinity binding sites are also present on fibroblasts expressing high amounts of TrkA in the absence of p75 (11), and Jing *et al.* (12) found that co-expression of p75 on fibroblasts expressing TrkA did not increase the number of high affinity binding sites. Importantly, more recent experiments have demonstrated the importance of a high p75 to TrkA ratio for the generation of NGF binding sites with the kinetic properties of the receptors seen in neurons and NGF-responsive cell lines (13).

The ligand-dependent autophosphorylation of tyrosine residues in the cytoplasmic domain of Trks clearly demonstrates that Trks can function as signaling receptors. In agreement with this, several studies have confirmed that Trks are both necessary and sufficient to mediate many of the effects elicited by neurotrophins (for a review see Ref. 14). Mice homozygous for null mutations in genes encoding neurotrophins and cognate Trks display striking similarities in their phenotypes, demonstrating that *in vivo* the neurotrophins mediate, to a large extent, their effects through activation of Trk receptors. Mutant animals display severe abnormalities in the nervous system, and most die shortly after birth, underlining the functional importance of these molecules during development. The p75 null mutant animals display a less severe phenotype, with p75 −/− mice living to several months of age but with reduced fertility. In addition mutant mice display significant abnormalities in developing sensory and sympathetic neurons (15). p75 has been proposed to be involved in retrograde transport of neurotrophins (16), ligand discrimination (17, 18), and ligand-dependent protection from, as well as induction of, apoptosis (19–23). Interestingly, recent *in vitro* studies have shown that binding of NGF to p75 can stimulate sphingomyelin hydrolysis (24) and activation of the transcription factor NFκB (25) in some non-neuronal cells in culture. p75 has also been suggested to play a modulatory role on signaling through TrkA. Absence of p75 or blocking of NGF binding to p75 reduces the responsiveness of several TrkA-mediated effects, including tyrosine autophosphorylation and cell differentiation (26, 27). Moreover, neurons from trigeminal and superior cervical ganglia of p75 −/− mice show a shifted NGF dose-response curve at later stages of development (28, 29). Taken together, results from immortalized cell lines and gene knock-out animals suggest that p75 affects NGF dosage sensitivity. While several models have been proposed to explain how p75 could function in this context, the molecular mechanism(s) is still unclear.

In the present study, we probed the possible roles of p75 on survival responses of normal neurons using a purified mutant NGF where p75 binding had been selectively abolished by mutation of exposed charged residues (30). These mutations, however, did not affect TrkA binding and activation. By comparing the biological effects elicited by native and mutant NGF on different responsive cells, the relative contribution of NGF-p75 interactions to NGF-dependent neuron survival can be investigated. Our results indicate that during development, p75 expression becomes increasingly important in regulating neuronal responsiveness to NGF. Expression of p75 could enable the individual neuron to respond adequately to low ligand concentrations or decreased receptor levels, thus improving the probability of survival in the developmental competition for trophic support.

MATERIALS AND METHODS

Cell Lines, Antisera, Proteins, and Plasmids—The generation of MG-87-NIH3T3 TrkA fibroblasts, kindly provided by George Yancopoulos, Regeneron Pharmaceuticals Inc., has previously been described (31). Anti-pan Trk and anti-p75 rabbit polyclonal antisera were a gift from David Kaplan, National Cancer Institute, Frederick, MD. A 771-base pair *Eco*RI fragment encoding mutant NGF (K32A + K34A + E35A) was introduced in baculovirus using a kit of reagents from CLONTECH. A recombinant virus clone was used for production of mutant NGF in insect cells, and protein was purified essentially as described previously (32). SDS-PAGE and silver staining showed that triNGF preparations displayed >90% purity.

Competitive and Saturation Binding Assays—Wild type NGF and triNGF were labeled with ^{125}I-Na (Amersham Corp.) by the lactoperoxidase method as described previously (33). Steady-state competitive bindings were performed at 4 °C as described previously (34). Saturation bindings were performed in triplicate wells with 10^6 cells/ml and serial dilutions of radioiodinated factors in Dulbecco's phosphate-buffered saline supplemented with 1 mg/ml bovine serum albumin and 1 mg/ml glucose. Cell pellets were counted in a gamma counter. Nonspecific binding was measured in parallel incubations to which a 3–500-fold molar excess of unlabeled wtNGF was added, and all results were corrected for this nonspecific binding.

Chemical Cross-linkings—MG-87-NIH3T3 TrkA cells or A875 human melanoma cells expressing high levels of p75 were affinity labeled with radioiodinated ligand and chemically cross-linked using either *N*-hydroxysuccinimidyl-4-azidobenzoate or 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (Pierce). Receptor complexes were immunoprecipitated overnight at +4 °C in the presence of proteinase inhibitors and separated by SDS-PAGE. Gels were dried and exposed to x-ray films with intensifying screens at −70 °C. Dissociated chick DRG neurons from different developmental stages were affinity labeled with 1 nM ^{125}I-NGF, followed by chemical cross-linking with sulfo-*N*-hydroxysulfosuccinimide and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (Pierce). 100 or 350 μg of protein (determined by the Bradford method) from each reaction was then separated by SDS-PAGE. Gels were dried and exposed to x-ray films with intensifying screens at −70 °C.

Isolation of RNA and RNase Protection Analysis—Chick dorsal root ganglia were homogenized in 4 M guanidine isothiocyanate and β-mercaptoethanol, and RNA was isolated as described previously. (35). Fragments derived from chick TrkA (kindly provided by Pantelis Tsoulfas, NCI) and chick p75 (36) were subcloned into pBS KS+ (Stratagene). Riboprobes were generated from linearized plasmids using either T3 or T7 RNA polymerase (Promega Biotech, Madison, WI) and [^{32}P]αCTP. Equal amounts of total RNA (5 μg) were analyzed by RNase protection assay according to manufacturer's instructions (Ambion Inc., Austin, TX). To control for differences in RNA loading a chick glyceraldehyde-3-phosphate dehydrogenase riboprobe (a kind gift from Finn Hallböök, Biomedicinskt Centrum, Uppsala, Sweden) labeled at low specific activity was added to each sample for subsequent standardization. Samples were separated in 5% polyacrylamide denaturing gels, dried, and exposed to x-ray films with intensifying screens at −70 °C. Autoradiograms were analyzed with an image analysis system (Leica); for quantification, all samples signals were standardized using the signal obtained with the glyceraldehyde-3-phosphate dehydrogenase probe.

Bioassays—Biological activities in TrkA-expressing MG87-NIH3T3 fibroblasts were assayed as described previously (34). Briefly, in a 96-well plate 20,000 cells/well were incubated in serial dilutions of native or mutant NGF in serum-free medium (Dulbecco's modified Eagle's medium). After a 4-day incubation, cellular acid phosphatase was measured using a kit of reagents from CLONTECH. Neurotrophin-mediated survival and proliferation were expressed in units of optical density measured at 405 nm. Dissociated neurons from dorsal root and superior cervical ganglia were cultured in Dulbecco's modified Eagle's medium/Ham's F12 (1:1) supplemented with 1 mg/ml bovine serum albumin, glutamine, and antibiotics. DRG cells were preplated on plastic for 2 h in order to enrich for neurons. 24-Well plates coated with poly-L-ornithine and laminin were seeded at a density of 10^5 cells/well in the presence of serial dilutions of factors. Neuronal survival was determined after 48–72 h by phase contrast microscopy, scoring the number of surviving neurons in a defined area of triplicate wells.

RESULTS

TriNGF Does Not Bind to p75 but Retains Binding to TrkA—A mutant NGF with Lys-32, Lys-34, and Glu-35 replaced by Ala (triNGF) was produced in baculovirus-infected

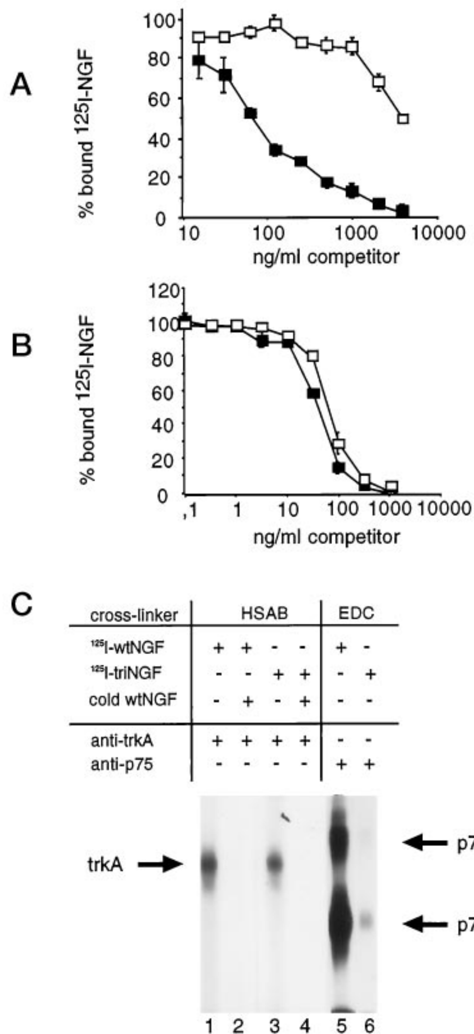

FIG. 1. **TriNGF lacks binding to p75 but retains wild-type binding to TrkA.** *A* and *B*, steady-state competitive binding assays were performed in the presence of ^{125}I-NGF and increasing concentrations of either wtNGF (*filled squares*) or triNGF (*open squares*) on cells expressing p75 (A875 human melanoma cells, *A*) or TrkA (MG-87-NIH3T3 TrkA, *B*). TriNGF displayed a 70-fold decrease in IC_{50} compared with wtNGF on p75 expressing cells but retained a virtually unimpaired binding to TrkA. *C*, radioiodinated wt- and triNGF were used to affinity label TrkA (MG-87-NIH3T3 TrkA, *lanes 1–4*) or p75 (A875 human melanoma cells, *lanes 5* and *6*) expressing cells. Ligand-receptor complexes were chemically cross-linked using *N*-hydroxysuccinimidyl-4-azidobenzoate or 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride, respectively, immunoprecipitated, separated by SDS-PAGE, and visualized by autoradiography. Where indicated cold NGF was used as a control for specific binding. *Arrows* indicate the migration of TrkA and p75 monomers and dimers, respectively.

Sf-21 insect cells and purified to homogeneity by a combination of ion exchange, size exclusion, and reverse phase chromatographies. The binding properties of purified triNGF to p75-expressing A875 melanoma cells and TrkA-expressing fibroblasts were in agreement with a previous report using protein from conditioned media of transiently transfected COS cells (30). The mutant showed a 70-fold reduction in binding to p75 expressed on A875 cells (wild type NGF IC_{50} 2.57 nM; triNGF IC_{50} 153 nM) (Fig. 1*A*), while binding to TrkA was comparable to native NGF (wild type NGF IC_{50} 1.53 nM; triNGF IC_{50} 2.43 nM) (Fig. 1*B*). Receptor binding was also assessed by affinity labeling and chemical cross-linking. Ligand-receptor complexes were immunoprecipitated using antisera against either TrkA or p75 and separated by SDS-PAGE. ^{125}I-NGF was efficiently cross-linked to TrkA expressed on 3T3-TrkA cells and to p75 monomers and dimers on A875 cells (Fig. 1*C, lane 1* and *5*). ^{125}I-triNGF was cross-linked to TrkA to a comparable extent (Fig. 1*C, lane 3*). However, triNGF bound 2 orders of magnitude less efficiently to p75 (Fig. 1*C, lane 6*), in agreement with the competition binding assays and confirming the TrkA selectivity of the mutant.

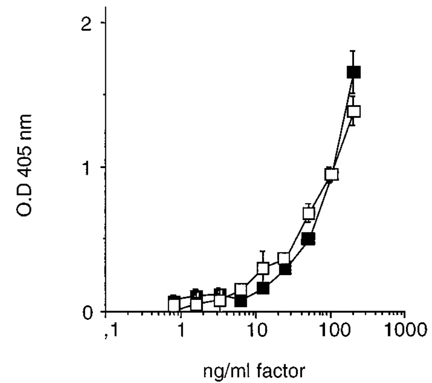

FIG. 2. **Binding of triNGF results in an unimpaired transduction of TrkA-mediated signals.** MG-87-NIH3T3 fibroblasts stably expressing TrkA were incubated for 4 days in serum-free cell media containing serial dilutions of wtNGF (*filled squares*) or triNGF (*open squares*). Cells survived and proliferated in a dose-dependent manner and were quantitated by an enzymatic method measuring cellular acid phosphatase (CLONTECH). Wild-type NGF and triNGF were equally potent in eliciting a proliferating response starting from 0.7 ng/ml.

Next, we compared the ability of native and mutant NGF to promote survival and growth of 3T3-TrkA fibroblasts. In serum-free medium, these cells can survive and proliferate in response to NGF, in a dose-dependent manner that is readily quantifiable. Wild type NGF and triNGF were equally effective at stimulating a proliferative response in these cells starting at 7 ng/ml (≈0.3 nM) (Fig. 2), showing that the binding of the mutant to TrkA resulted in normal receptor activation and downstream signaling.

TriNGF Displays Only Low Affinity Binding to PC-12 Cells—We next investigated whether loss of p75 binding affected the interaction of triNGF with high and low affinity binding sites on PC12 cells expressing both p75 and TrkA receptors. Saturation binding assays were performed at equilibrium using purified radioiodinated ligands. In agreement with previous reports (37), Scatchard transformation of the data showed that native NGF bound to two distinct classes of binding sites, a high affinity site of K_d 3×10^{-11} M and a low affinity site of K_d 3×10^{-9} M (Fig. 3*A*). In contrast, ^{125}I-triNGF displayed only low affinity binding with K_d 4.3×10^{-9} M (Fig. 3*B*), suggesting that interaction with high affinity receptors requires NGF binding to p75.

Lack of Binding to p75 Attenuates NGF-mediated Survival Responses in Primary Sensory Neurons at Low Ligand Concentrations—In an effort to clarify the role of p75 in physiologically relevant cells, we studied the effects of wild type and mutant NGF on primary cultures of NGF-responsive neurons. We reasoned that given the comparable TrkA activation profile of the two ligands, differences in biological activity could be attributed to the inability of triNGF to bind to p75. We compared survival-promoting activities of native and mutant NGF in dissociated cultures of embryonic day 9 (E9) chicken dorsal root ganglion (DRG) neurons expressing both p75 and TrkA receptors. In this assay, triNGF showed a close to wild type dose-response relationship at concentrations above 0.3 ng/ml (≈12 pM) (Fig. 4). Below this concentration, however, the mutant NGF was clearly impaired in its biological potency; at 40 pg/ml (≈1.5 pM), triNGF rescued less than 10% of the neurons sur-

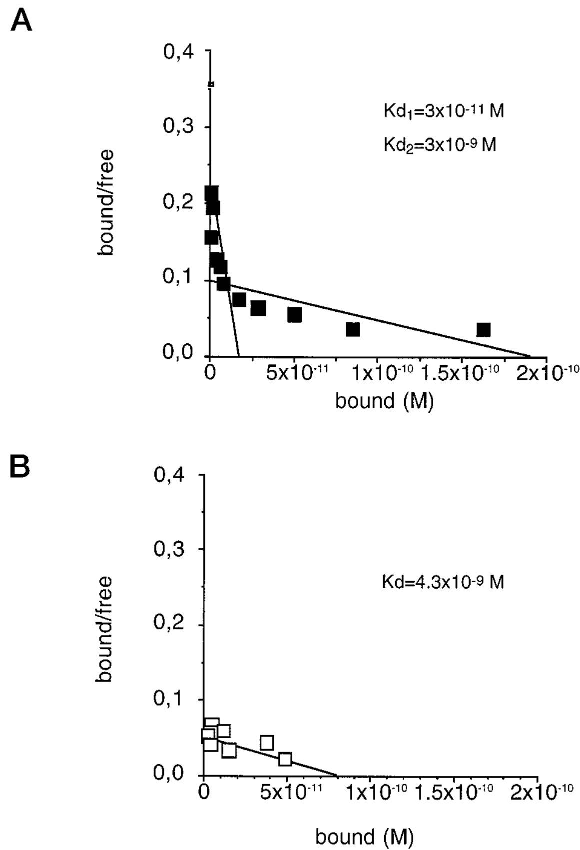

FIG. 3. **TriNGF does not interact with high affinity binding sites on cells co-expressing TrkA and p75.** Scatchard transformations of saturation steady-state bindings with radioiodinated wtNGF (*A*) or triNGF (*B*) on PC12 cells. Wild-type NGF bound to two distinct binding sites with calculated K_d values in the picomolar and nanomolar range, respectively. In contrast, triNGF displayed only low affinity binding with a K_d in the nanomolar range.

viving in the presence of equivalent concentrations of native NGF. The shift in responsiveness occurred at concentrations corresponding to the dissociation constant of high affinity binding of NGF to these cells (37) and correlated with the lack of high affinity binding of the mutant to PC12 cells (Fig. 3*B*).

Reduced Responsiveness to triNGF during Development—Previous studies have shown a reduced survival response to NGF during development in neurons of p75 −/− mice (28, 29). However, those studies could not establish whether NGF responsiveness was dependent on the mere presence of p75 on the cell membrane or whether NGF binding to p75 was also required. In addition, they could not rule out differences in the neurons secondary to the loss of p75. We investigated the responsiveness of chicken DRG neurons prepared from embryos of different developmental stages ranging from E7 to E18 to a concentration of native and mutant NGF (5 ng/ml) which was saturating at early times of development (see Fig. 4). At early embryonic stages (E7–E11), triNGF was comparable to wtNGF; both proteins were able to rescue close to 100% of the plated neurons at 5 ng/ml. At later developmental stages, increasing numbers of DRG neurons became NGF-independent and survived in the absence of any added factor (Fig. 5*A, solid circles*). At E18, 50% of the neurons plated still depended on exogenous NGF for survival. However, the reduction in responsiveness was dramatic for the mutant NGF; at E14 30–40% less of the plated neurons responded to this molecule compared with native NGF, and at later stages, the response to triNGF was virtually null (Fig. 5, *A* and *B*). Thus, the mutant NGF displays a substantially reduced bioactivity at later stages in

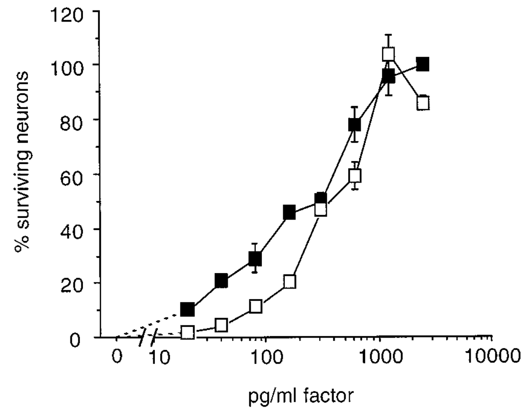

FIG. 4. **The responsiveness of sensory neurons to triNGF is compromised at low ligand concentrations.** E9 chick DRG neurons were dissociated and incubated in triplicate wells containing serial dilutions of wtNGF (*filled squares*) or triNGF (*open squares*). Neuronal survival was determined after 72 h of incubation by counting the number of surviving neurons in defined areas of the wells. Results are expressed as the percentage of surviving cells relative to the number scored in the presence of the maximal concentration of wtNGF (3 ng/ml). *Error bars* indicate ± S.D. Although the two factors were indistinguishable in promoting neuronal survival above 300 pg/ml, triNGF showed a clear reduction in biological activity at levels below this.

DRG development, indicating that not only expression of but also binding to p75 is required for normal NGF responsiveness during sensory neuron development.

We also studied sympathetic neurons from the superior cervical ganglion (SCG) of the rat, which undergo naturally occurring cell death during early postnatal development (38). Survival dose-response relationships were studied for wild type and mutant NGF at two developmental stages, E16.5 and postnatal day 2 (P2). At E16.5, no difference in potency or efficacy could be observed between mutant and native NGF, even at low concentrations (Fig. 6*A*). However, on P2 SCG neurons, a clear reduction in biological activity was detected for the mutant NGF at concentrations below 1 ng/ml (Fig. 6*B*), again indicating that binding of NGF to p75 is essential for survival of NGF-dependent peripheral neurons during later stages of development.

The Decreased Developmental Responsiveness to triNGF Correlates with Reduced Expression Levels of p75 and TrkA in Chick DRG Neurons—p75 and TrkA mRNA levels were measured by RNase protection assay in chick DRG neurons at two different stages of development marking the beginning and the ending of the switch in responsiveness to the mutant NGF, *i.e.* E8 and E16, respectively. Prior to homogenization, DRG cells were dissociated, and neurons were enriched by pre-plating in uncoated plastic dishes. The levels of mRNA expression of both TrkA and p75 in chick DRG neurons were found to decrease from E8 to E16 (Fig. 7, *A* and *B*). The decrease in TrkA and p75 mRNA levels was accompanied by a corresponding reduction in the levels of surface receptors as demonstrated by affinity labeling with ^{125}I-NGF followed by chemical cross-linking (Fig. 7*C*). Despite the developmental reduction in the absolute levels of NGF receptors, the relative levels of expression of TrkA and p75 did not show a significant change. In rodent SCG neurons, on the other hand, p75 and TrkA mRNA levels have been reported to increase during development (39), although, in this case, a relative increase in the proportion of p75 mRNA with respect to TrkA was observed (39). Thus, the contribution of p75 to neuron survival becomes more significant in neurons undergoing a down-regulation of NGF receptors or when the expression of p75 increases relative to that of TrkA.

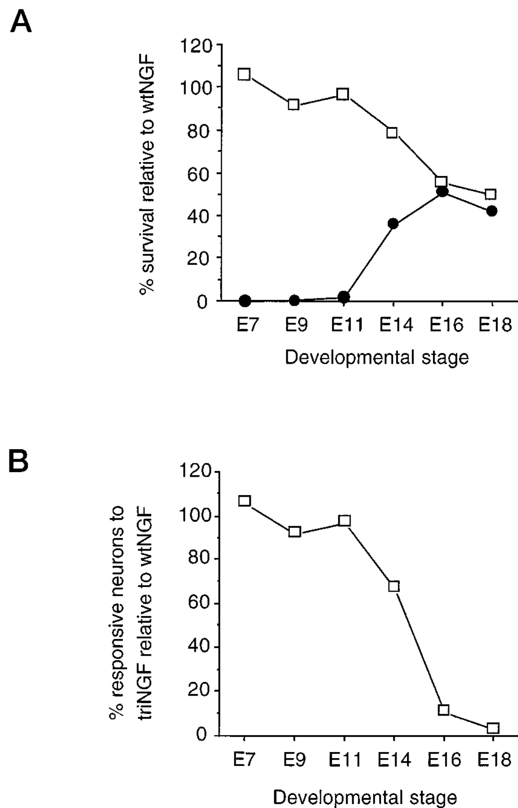

FIG. 5. **The responsiveness of sensory neurons to triNGF is decreased during development.** *A*, chick DRG neurons from the indicated stages of embryonic development were incubated with either serum-free medium only (*solid circles*) or medium containing 5 ng/ml wtNGF or triNGF (*open squares*). This concentration was previously shown to be saturating for both factors at E9 (Fig. 4). The number of neurons surviving with wtNGF at each stage was set to represent 100%. Neuronal survival was scored, and the results are expressed as the percentage of surviving neurons relative to wtNGF-incubated wells. Note that the number of NGF-nondependent neurons increases with development (*solid circles*). More importantly, the responsiveness to triNGF is decreased from E14 and onward. *B*, relative neuronal rescue by triNGF compared with wtNGF is expressed after subtracting the control values in *A*. Note that at E16 virtually none of the NGF-responsive neurons are supported by triNGF. Results display the mean of two independent triplicate experiments.

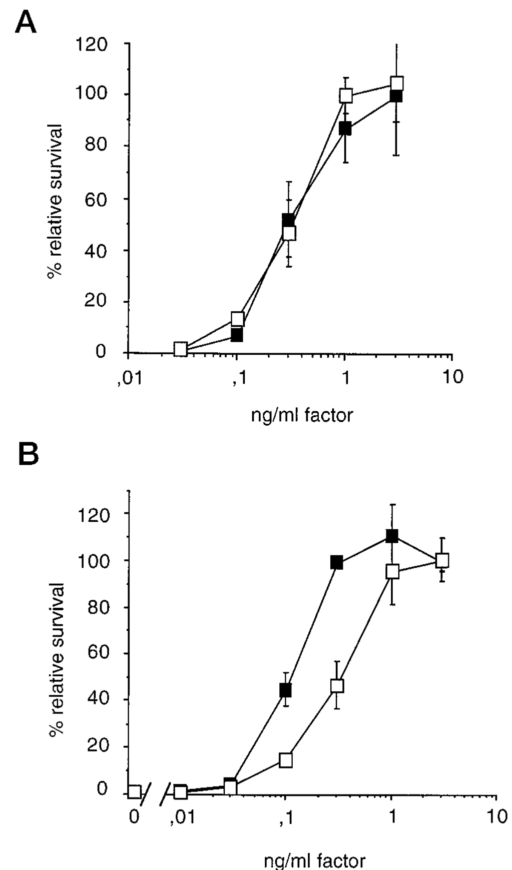

FIG. 6. **The responsiveness of sympathetic neurons to triNGF is decreased during development.** Dissociated sympathetic neurons from the superior cervical ganglion of E16.5 (*A*) and P2 (*B*) rats were incubated in the presence of wtNGF (*filled squares*) or triNGF (*open squares*) in triplicate wells. After 48 h the number of surviving cells were counted in defined areas of the wells. Results are expressed as the percentage of neurons surviving relative to the number surviving in the highest concentration of wtNGF (3 ng/ml). *Error bars* indicate ± S.D. At E16.5, no difference could be detected between the two ligands. However, in P2 neurons, triNGF was clearly impaired in its ability to promote survival.

DISCUSSION

We have used neurotrophin analogues generated by site-directed mutagenesis as a tool to assess functional roles of p75 and Trk receptors. Neurotrophins that bind selectively to one class of receptor make it possible to delineate the ligand-activated contribution of each receptor to different biological responses (for a recent review see Ref. 40). To determine the importance of NGF binding to p75 in neuron survival, we have used a mutant NGF that lacks significant p75 binding while retaining TrkA binding and activation. An important conclusion from these results is that the NGF binding to p75 modulates neuronal responsiveness to this neurotrophin. This was, however, only observed when the ligand concentration was low. Comparison of the biological activity of triNGF with native NGF on sensory neurons showed that, although survival-promoting activities were identical at ligand concentrations exceeding 0.03 nM (1 ng/ml), the mutant was clearly impaired below this level. We think that it is unlikely that these differences in activity could be accounted for by differences in the relative abilities of wild type and mutant NGF to homodimerize at very low concentrations. The residues mutated in triNGF are highly exposed and not directly compromised in either intra- or inter-protomer interactions; moreover, the replacements do not appear to affect the stability of the molecule (30). Together, the results presented in this study indicate that at concentrations corresponding to the levels of NGF found *in vivo* (5), ligand binding to p75 augments the survival response. Furthermore, our data reveal alterations in this response during development, such that amounts of triNGF that were saturating at early developmental stages had no effect on neuron survival at later stages. To correlate NGF responsiveness with receptor expression, we measured p75 and TrkA mRNA levels during sensory neuron development. We found that p75 and TrkA mRNA levels were down-regulated in developing DRG, suggesting that the decreased responsiveness to triNGF seen in chick DRG neurons during development is a consequence of the down-regulation of NGF receptors. It would then appear that just as binding to p75 is important for responsiveness at low ligand concentrations, it is also important for responsiveness in situations of reduced receptor expression. On the other hand, NGF receptor expression has been shown to increase during late embryonic and early post-natal SCG development (39). Using reverse transcriptase-polymerase chain reaction analysis, these authors demonstrated that the ratio of p75 to TrkA mRNA expression increases with development of SCG neurons (39), and these data have been taken as an argument to explain the developmental switch in responsiveness to NGF observed in SCG from mice lacking p75 (28). It is therefore

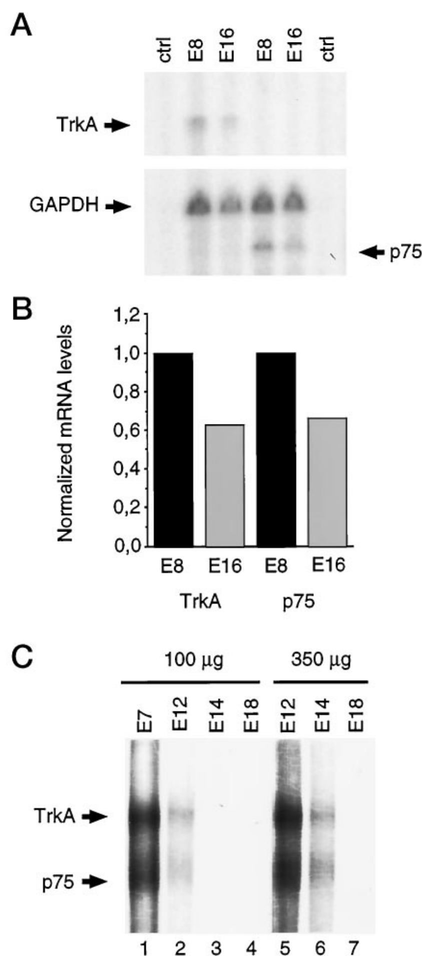

FIG. 7. **Decreased levels of expression of p75 and TrkA in developing chick DRG neurons.** *A*, total RNA was isolated from neuron-enriched chick DRG cells at two different embryonic stages, E8 and E16, and analyzed by RNase protection assay with riboprobes specific for chick TrkA and p75. *B*, histogram showing the PhosphorImager quantification of the image shown in *A*. TrkA and p75 mRNA levels were standardized to the signal obtained with a low specific activity chick glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) riboprobe added in the same reaction. Similar results were obtained in a duplicate experiment. *C*, affinity labeling of NGF receptors in neuron-enriched chick DRG cells confirms the developmental reduction in the levels NGF of surface receptors. *Lanes 1–4,* 100 µg of protein loaded; *lanes 5–7,* 350 µg of protein loaded. Bands corresponding to affinity labeled TrkA and p75 are indicated.

possible that the decreased responsiveness to triNGF in developing SCG is due to a shift in the p75/trkA expression ratio.

Using a similar approach, we have previously shown that p75 plays a role in modulating NT-4 signaling. Point mutations in BDNF, NT-3, and NT-4 in the equivalent residues altered in triNGF also resulted in neurotrophins that lacked p75 binding but retained binding and activation of cognate Trks. In cells co-expressing p75 and Trks, the mutated NT-4, but not mutant BDNF or NT-3, displayed a markedly reduced biological activity (32), similar to the results reported here with triNGF. These data, together with the finding that p75 binds all neurotrophins with different kinetic properties (2), indicate a differential role of p75 in neurotrophin signaling. Whether in some cases p75 may also modulate the activities of BDNF and NT-3 remains to be demonstrated.

How do our results compare with previous reports that failed to document a role for p75 in the response to NGF? Jing *et al.* (12) could not detect any difference in NGF-induced tyrosine phosphorylation of TrkA in fibroblasts overexpressing this receptor in the presence or absence of p75. However, in this study, both receptors were expressed at equimolar levels, and furthermore, cell-surface TrkA protein was expressed at high levels compared with those found in PC-12 cells and neurons. In this respect, our data, together with results obtained at other laboratories, indicate that the modulatory role of p75 is easily saturable and depends upon the relative level of p75 with respect to TrkA and the concentration of NGF. In another study, no alteration in NGF-mediated survival or neurite outgrowth could be seen in early embryonic DRG neurons or PC12 cells in the presence of antibodies that blocked NGF binding to p75 (41). However, the concentrations of NGF used in those assays were all in the nanomolar range, well above the level at which the modulatory effects of p75 could be detected in our experiments.

How does p75 modulate NGF signaling? In one proposed model, NGF first binds rapidly to p75, and due to the fast dissociation rate from this receptor, NGF is then delivered to TrkA in a favorable conformation for binding. A variant of this model proposes a concentrative role for p75, whereby NGF binding to this receptor results in an increased NGF concentration in the vicinity of TrkA. Neither of these models requires a direct interaction between p75 and TrkA and could in principle involve p75 molecules present on the membrane of responsive (cis) or adjacent (trans) cells. Analogous models have been proposed for other growth factor receptor complexes, including receptors for transforming growth factor-βs (42) and for tumor necrosis factor (43). In contrast, the conformational model proposes that p75 expression alters the conformation of TrkA to bind NGF with higher affinity and/or to increase signal transducing capability and predicts a direct interaction between TrkA and p75 in the absence of ligand. Interestingly, Huber and Chao (44) have presented evidence for the existence of p75-TrkA complexes by co-immunoprecipitation of affinity-labeled receptors in sensory neurons. Also recently, Ross *et al.* (45) used a co-patching technique in insect cells overexpressing p75 and TrkA to show that these two receptors may interact directly, forming receptor clusters even in the absence of ligand, However, molecular evidence for a change in TrkA conformation resulting in a higher affinity binding state has yet to be demonstrated. Because triNGF bound to TrkA with near wt affinity in the absence of p75, for our results to be compatible with the conformational model, p75 would have to change TrkA into a receptor to which triNGF can no longer bind. Although we cannot at present evaluate the likelihood of this possibility, the conformational model would then predict the mutated residues in triNGF to be indispensable for the interaction of NGF with a novel TrkA high affinity binding site, in addition to their demonstrated role in p75 binding.

Finally, the possibility that p75 modulates NGF signaling and neuron survival by directly activating an intracellular pathway should not be ruled out. Very recently, four independent reports have indicated that activation of the transcription factor NFκB inhibits apoptosis induced by the tumor necrosis factor receptor (46–48), but not by Fas (49, 50), which does not result in NFκB activation. NGF binding to p75 has been shown to result in the activation and nuclear translocation of NFκB in p75-expressing fibroblasts and in Schwann cells, and this receptor has been proposed to be involved in ligand-dependent induction (21, 22) as well as inhibition (20) of apoptosis. As in the case of the tumor necrosis factor receptor, these seemingly contradictory effects could be reconciled if NGF binding to p75 activates parallel signaling pathways leading to cell killing and cell survival in specific cellular contexts. Intriguingly, NGF-induced ceramide production as well as NFκB activation have so far only been observed in cells expressing p75 in the absence of detectable levels of TrkA (24, 25, 51).

In conclusion, our results indicate that ligand binding to p75 augments responsiveness to NGF, particularly when NGF is present at limiting concentrations. During development, p75 modulates responsiveness to NGF so that binding to p75 becomes increasingly important in neurons undergoing a down-regulation of NGF receptors. These data support a ligand-dependent modulatory role for p75 in NGF-mediated neuron survival consistent with p75 functioning as a TrkA regulator and/or signaling receptor.

Acknowledgments—We thank Dr. Mike Fainzilber for constructive comments on the manuscript. We also thank Ann-Sofie Nilsson and Debbie Mahadeo for excellent technical assistance, Pellina Janson for help with animal care, Lotta Johansson for secretarial work, and Annika Ahlsén for additional help.

REFERENCES

1. Bothwell, M. (1995) *Annu. Rev. Neurosci.* **18,** 223–253
2. Rodriguez-Tébar, A., Dechant, G., Gotz, R., and Barde, Y. A. (1992) *EMBO J.* **11,** 917–922
3. Sutter, A., Riopelle, R. J., Harris-Warrick, R. M., and Shooter, E. M. (1979) *J. Biol. Chem.* **254,** 5972–5982
4. Schechter, A. L., and Bothwell, M. A. (1981) *Cell* **24,** 867–874
5. Meakin, S. O., and Shooter, E. M. (1992) *Trends Neurosci.* **15,** 323–331
6. Barde, Y. A., Edgar, D., and Thoenen, H. (1982) *EMBO J.* **1,** 549–553
7. Rodriguez-Tébar, A., Dechant, G., and Barde, Y. A. (1990) *Neuron* **4,** 487–492
8. Meakin, S. O., Suter, U., Drinkwater, C. C., Welcher, A. A., and Shooter, E. M. (1992) *Proc. Natl Acad. Sci. U. S. A.* **89,** 2374–2378
9. Hempstead, B. L., Martin-Zanca, D., Kaplan, D. R., Parada, L. F., and Chao, M. V. (1991) *Nature* **350,** 678–683
10. Kaplan, D. R., Hempstead, B. L., Martin-Zanca, D., Chao, M. V., and Parada, L. F. (1991) *Science* **252,** 554–556
11. Klein, R., Jing, S., Nanduri, V., O'Rourke, E., and Barbacid, M. (1991) *Cell* **65,** 189–197
12. Jing, S., Tapley, P., and Barbacid, M. (1992) *Neuron* **9,** 1067–1079
13. Mahadeo, D., Kaplan, L., Chao, M. V., and Hempstead, B. L. (1994) *J. Biol. Chem.* **269,** 6884–6891
14. Lewin, G. R., and Barde, Y. A. (1996) *Annu. Rev. Neurosci.* **19,** 289–317
15. Lee, K.-F., Li, E., Huber, L. J., Landis, S. C., Sharpe, A. H., Chao, M. V., and Jaenisch, R. (1992) *Cell* **69,** 737–749
16. Johnson, E. M. J., Taniuchi, M., Clark, H. B., Springer, J. E., and Koh, S. (1987) *J. Neurosci.* **7,** 923–929
17. Clary, D. O., and Reichard, L. F. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 11133–11137
18. Benedetti, M., Levi, A., and Chao, M. V. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90,** 7859–7863
19. Van der Zee, C., Ross, G. M., Riopelle, R. J., and Hagg, T. (1996) *Science* **274,** 1729–1732
20. Rabizadeh, S., Oh, J., Zhong, L., Yang, J., Bitler, C. M., Butcher, L. L., and Bredesen, D. E. (1993) *Science* **261,** 345–348
21. Frade, J. F., Rodriguez-Tébar, and Barde, Y. A. (1996) *Nature* **383,** 166–168
22. Casaccia-Bonnefil, P., Carter, B. D., Dobrowsky, R. T., and Chao, M. V. (1996) *Nature* **383,** 716–719
23. Barret, G. L., and Bartlett, P. F. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 6501–6505
24. Dobrowsky, R. T., Werner, M. H., Castellino, A. M., Chao, M. V., and Hannun, Y. A. (1994) *Science* **265,** 1596–1599
25. Carter, B. D., Kaltschmidt, C., Kaltschmidt, B., Offenhäuser, N., Böhm-Mattei, R., Baeuerle, P., and Barde, Y. A. (1996) *Science* **272,** 542–545
26. Verdi, J. M., Birren, S. J., Ibáñez, C. F., Persson, H., Kaplan, D. R., Benedetti, M., Chao, M. V., and Anderson, D. J. (1994) *Neuron* **12,** 733–745
27. Barker, P. A., and Shooter, E. M. (1994) *Neuron* **13,** 203–215
28. Lee, K. L., Davies, A. M., and Jaenisch, R. (1994) *Development* **120,** 1027–1033
29. Davies, A. M., Lee, K. L., and Jaenisch, R. (1993) *Neuron* **11,** 565–574
30. Ibáñez, C. F., Ebendal, T., Barbany, G., Murray-Rust, J., Blundell, T. L., and Persson, H. (1992) *Cell* **69,** 329–341
31. Glass, D. J., Nye, S. H., Hantzopoulos, P., Macchi, M. J., Squinto, S. P., Goldfarb, M., and Yancopoulos, G. D. (1991) *Cell* **66,** 405–413
32. Rydén, M., Murray-Rust, J., Glass, D., Ilag, L. L., Trupp, M., Yancopoulos, G. D., McDonald, N. Q., and Ibáñez, C. F. (1995) *EMBO J.* **14,** 1979–1990
33. Ibáñez, C. F., Ilag, L. L., Murray-Rust, J., and Persson, H. (1993) *EMBO J.* **12,** 2281–2293
34. Rydén, M., and Ibáñez, C. F. (1996) *J. Biol. Chem.* **271,** 5623–5627
35. Trupp, M., Rydén, M., Jörnvall, H., Timmusk, T., Funakoshi, H., Arenas, E., and Ibáñez, C. F. (1995) *J. Cell Biol.* **130,** 137–148
36. Ernfors, P., Hallböök, F., Ebendal, T., Shooter, E. M., Radeke, M. J., Misko, T. P., and Persson, H. (1988) *Neuron* **1,** 983–996
37. Green, S. H., Rydel, R. E., Connolly, J. L., and Greene, L. A. (1986) *J. Cell Biol.* **102,** 830–843
38. Wright, L. L., Cunninham, T. J., and Smolen, A. J. (1983) *J. Neurocytol.* **12,** 727–738
39. Wyatt, S., and Davies, A. M. (1995) *J. Cell Biol.* **130,** 1435–1446
40. Ibáñez, C. F. (1995) *Trends Biotechnol.* **13,** 217–227
41. Weskamp, G., and Reichard, L. F. (1991) *Neuron* **6,** 649–663
42. López-Casillas, F., Wrana, J. L., and Massagué, J. (1993) *Cell* **73,** 1435–1444
43. Tartaglia, L. A., Pennica, D., and Goeddel, D. V. (1993) *J. Biol. Chem.* **268,** 18542–18548
44. Huber, L. J., and Chao, M. V. (1995) *J. Neurosci. Res.* **40,** 557–563
45. Ross, A. H., Daou, M. C., McKinnon, C. A., Condon, P. J., Lachyankar, M. B., Stephens, R. M., Kaplan, D. R., and Wolf, D. E. (1996) *J. Cell Biol.* **132,** 945–953
46. van Antwerp, D. J., Martin, S. J., Kafri, T., Green, D. R., and Verma, I. M. (1996) *Science* **274,** 787–789
47. Beg, A. A., and Baltimore, D. (1996) *Science* **274,** 782–784
48. Wang, C. Y., Mayo, M. W., and Baldwin, A. S. J. (1996) *Science* **274,** 784–787
49. Nagata, S., and Golstein, P. (1995) *Science* **267,** 1449–1456
50. Liu, Z., Hsu, H., Goeddel, D. V., and Karin, M. (1996) *Cell* **87,** 565–576
51. Dobrowsky, R. T., Jenkins, G. M., and Hannun, Y. A. (1995) *J. Biol. Chem.* **270,** 22135–22142