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. The mutant NGF (triNGF) deficient in p75 binding but retaining 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. The mutant was found to be as potent as wild type NGF at promoting survival of serum-deprived TrkA-expressing fibroblasts. The mutant was found to be as potent as wild type NGF at promoting survival of serum-deprived TrkA-expressing fibroblasts. The mutant was found to be as potent as wild type NGF at promoting survival of serum-deprived TrkA-expressing fibroblasts.

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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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, 125I-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 domain 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 saturating at low concentrations at nanomolar concentrations. However, triNGF was 3- to 4-fold less potent than wild type NGF at nanomolar concentrations. How-sponses 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 nanomolar 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.

* 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 Hirsch/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.
demonstrating that nate Trks display striking similarities in their phenotypes, for null mutations in genes encoding neurotrophins and cog-

by neurotrophins (for a review see Ref. 14). Mice homozygous with this, several studies have confirmed that Trks are both

that Trks can function as signaling receptors. In agreement
duases in the cytoplasmic domain of Trks clearly demonstrates

(13).

Jing

pressing high amounts of TrkA in the absence of p75 (11), and

high affinity binding sites are also present on fibroblasts ex-

formation of high affinity binding sites. In contrast, equilib-

low affinity, whereas co-expression of both receptors results in

binding of NGF to p75 can stimulate sphingomyelin hydrolysis

Moreover, neurons from trigeminal and superior cervical gan-

gest that p75 affects NGF dosage sensitivity. While several

somes non-neuronal cells in culture. p75 has also been sug-
gests the importance of a high p75 to TrkA ratio for the

some non-neuronal cells in culture. p75 has also been sug-
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expression becomes increasingly important in regulating neuronal responsiveness to NGF. Expression of p75 could en-
able 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

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, equilibr-

binding analysis demonstrated that a small number of high affinity binding sites are also present on fibroblasts ex-

pressing 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 dem-

strated the importance of a high p75 to TrkA ratio for the

number 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 resi-
dues 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 cogn-

ate 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 func-
tional importance of these molecules during development. The p75 null mutant animals display a less severe phenotype, with

5/—/ mice living to several months of age but with reduced fertility. In addition mutant mice display significant abnormal-

ities 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 sphenogomyelin hydrolysis (24) and activation of the transcription factor NFkB (25) in

some non-neuronal cells in culture. p75 has also been sug-
gested 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 ty-

rosine autophosphorylation and cell differentiation (26, 27). Moreover, neurons from trigeminal and superior cervical gan-

glia 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 sug-

gest 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 com-

paring 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 en-
able 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.
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 125I-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 IC50 compared with wtNGF on p75 expressing cells but retained a virtually unimpaired binding to TrkA. C, radiiodinated 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.

p75 Modulates Neuronal Survival

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 radiiodinated 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 Kd1 = 3 × 10−11 M and a low affinity site of Kd2 = 3 × 10−8 M (Fig. 3A). In contrast, 125I-triNGF displayed only low affinity binding with Kd = 4.3 × 10−8 M (Fig. 3B), 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-
binding with a range, respectively. In contrast, triNGF displayed only low affinity to a concentration of native and mutant NGF (5 ng/ml) which was saturating at early times of development (see Fig. 4). At E18, 50% of the neurons plated still depended on exogenous NGF for survival. However, the reduction 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. 3B). 

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. 5A, 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 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. 6A). However, on P2 SCG neurons, a clear reduction in biological activity was detected for the mutant NGF at concentrations below 1 ng/ml (Fig. 6B), 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 [125I]-NGF followed by chemical cross-linking (Fig. 7C). 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.

![Graph](https://via.placeholder.com/150)

**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 radiolabeled 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.

**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.
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
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-β and for tumor necrosis factor. 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 Ahlésén for additional help.

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