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Differential expression of nerve growth factor receptors leads to altered binding affinity and neurotrophin responsiveness

(Trk receptor/neurotrophin 3/high-affinity binding)

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ABSTRACT **The low-affinity p75 neurotrophin receptor is believed to participate with the Trk receptor tyrosine kinase in the formation of high-affinity binding sites for nerve growth factor (NGF). To investigate the functional significance of the two NGF receptors, a truncated p75 receptor was stably expressed in PC12 rat pheochromocytoma cells, yielding cells with greatly reduced levels of wild-type p75 and normal Trk levels. Although these cells were capable of normal differentiation by NGF, very few high-affinity NGF binding sites were detected. These findings indicate that high-affinity binding may be functionally dissociated from biological responses. Furthermore, an increased responsiveness to neurotrophin 3 was observed, as manifested by increased neurite outgrowth. These results suggest that a correct ratio of p75 and p140^{trk} is required to create high-affinity sites and that p75 expression may assist in the discrimination between related but different neurotrophin factors.**

Neurotrophins, such as nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin 3 (NT-3), NT-4, and NT-5, are required for the survival of overlapping but distinct populations of neurons (1, 2). These trophic factors constitute a family that interacts with two different receptor classes, the Trk receptor tyrosine kinase family (3, 4) and the p75 neurotrophin receptor, originally defined as a NGF receptor (p75^{NGFR}) by gene-transfer experiments (5, 6). The Trk receptor family transduces neurotrophin signaling through autophosphorylation and increased tyrosine phosphorylation of cellular proteins.

Neurotrophins are capable of binding independently to each receptor type. NGF interacts directly with p140^{trkA}, whereas BDNF and NT-3 bind and activate most preferentially p145^{trkB} and p145^{trkC}, respectively (7, 8). The p75 receptor can bind specifically to BDNF and NT-3 (9, 10) and probably serves as a receptor for all members of the neurotrophin factor family. Reconstitution studies imply that p140^{trk} and p75 are necessary for formation of high-affinity NGF binding sites (11). High-affinity binding has also been observed with p140^{trk} in the absence of p75 (12, 13), giving rise to different models of neurotrophin receptor action (7, 8).

The precise functions of the p75 neurotrophin receptor in NGF-mediated signaling are not fully understood. A number of experiments have indicated that p75 is not necessary for signaling. For example, Trk tyrosine kinase activation can result in a proliferative response in the absence of p75 in fibroblasts (14) and in a differentiative outcome in sympathoadrenal precursor cells after membrane depolarization (15). Use of p75 antibodies and mutant recombinant versions of NGF have implied that NGF action can be mediated through p140^{trk}, without binding to the p75 receptor (16, 17). However, a targeted mutation in the gene encoding p75 resulted in mutant mice with dramatic sensory deficiencies, such as heat insensitivity (18), indicating that p75 plays an essential role in NGF function.

Both p75 and p140^{trk} are coexpressed *in vivo* in the majority of NGF-responsive neuronal populations, such as sensory and sympathetic neurons, and cholinergic neurons in the basal forebrain (19–21). An essential question that arises is why neurotrophins interact with these two distinct receptor molecules. We have approached this question by studying a mutant PC12 rat pheochromocytoma cell line which is highly deficient in wild-type p75. These cells express a truncated receptor carrying the transmembrane and cytoplasmic domains of p75. Here we report that these cells display striking differences in high-affinity binding and responsiveness toward related neurotrophin factors.

MATERIALS AND METHODS

Cells and Reagents. PC12 cells were cotransfected on 100-mm dishes with the mR plasmid and pSV2neo at a 10:1 ratio by the Lipofectin procedure (BRL). Independent clones were isolated after growth and selection with the neomycin analogue G418 (GIBCO) at 0.5 mg/ml. Antiserum to NGF was from Collaborative Research. NGF was from Bioproducts for Science (Indianapolis), and NT-3 was a gift from Chiron. ^{125}I-labeled NGF was prepared by lactoperoxidase treatment and used within 10 days. Crosslinking reactions with 3-ethyl-1-(3-dimethylaminopropyl)carbodiimide (EDAC, Pierce) and disuccinimidyl suberate (DSS, Pierce) were carried out as described (11, 22, 23).

Construction of Truncated p75 Receptor. The expression vector utilized the RSVneo plasmid (24). The sequence coding for neomycin resistance was replaced by the mR p75 cDNA construct. The hybrid mR gene encoded the leader sequence and the first 25 aa from the λ immunoglobulin chain (25) fused in frame to 12 aa of the human c-Myc epitope recognized by the 9E10 monoclonal antibody (26). This leader sequence was subcloned next to a human cDNA for p75 beginning at aa 215 (27). This truncated cDNA was acquired by deleting between the *Nco* I (nt 139) and *Bst*EII (nt 838) sites, effectively removing the signal peptide and all but 8 aa N-terminal to the transmembrane domain and ending at the *Bst*EII site at aa 215. The mR cDNA was driven by the Rous sarcoma virus promoter.

Immunoblot Analysis. Cells were grown to near confluence on 150-mm dishes, lysed, and subjected to immunoprecipitation (where noted) and to Western blot analysis (28). Nitrocellulose filters were incubated with anti-p75 antibodies, followed by ^{125}I-labeled-protein A (Amersham). The antiserum against the cytoplasmic domain of p75 was generated against a glutathione *S*-transferase fusion protein (no.

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Abbreviations: NGF, nerve growth factor; BDNF, brain-derived neurotrophic factor; NT-*n*, neurotrophin *n*; EDAC, 3-ethyl-1-(3-dimethylaminopropyl)carbodiimide; DSS, disuccinimidyl suberate.

9992) containing aa 276–399 of the human p75 (29). A similar antibody was a gift from S. Decker (Parke–Davis).

NGF Binding. A filter binding assay was used on membrane preparations (11). Nonspecific binding was assessed by including excess unlabeled NGF. Specific binding ranged from 50% to 80% of total binding. All binding reactions (total and nonspecific) were carried out in triplicate.

RNA Analysis. Hybridization of total RNAs separated in 2.2 M formaldehyde/agarose gels took place on nitrocellulose filters in 5× SSC/50% formamide at 42°C. Probes specific for TrkA, TrkB, and TrkC mRNAs, and 3T3 cell lines expressing each Trk family member, were generously provided by Pantelis Tsoulfas, Dan Soppet, and Luis Parada (National Cancer Institute, Frederick, MD). RNA was isolated by homogenization in guanidinium thiocyanate solution and CsCl centrifugation.

RESULTS

Generation of Cell Lines. PC12 cell lines that exhibited normal levels of $p140^{trk}$, but very low levels of p75, were identified in gene-transfer experiments. These cell lines were an unexpected consequence of transfection with a cDNA encoding a truncated receptor with the transmembrane and cytoplasmic domains of p75. Fig. 1*A* depicts the wild-type p75 structure and the deletion construct, referred to as mR. The mR receptor is not capable of ligand binding. To facilitate detection of this truncated receptor, a c-Myc epitope was inserted which is recognized by the monoclonal antibody 9E10 (26).

Cell lines were screened for expression of the mR protein by indirect immunofluorescence experiments with the 9E10 antibody. The mR gene product was observed predominantly at the cell surface, indicating a correct processing and targeting of the protein. Cell lines were examined by immunoblotting using the anti-Myc antibody 9E10 and polyclonal antiserum to the intracellular domain of p75. The mR protein was detected both by antibody against the Myc epitope and by antiserum against the p75 cytoplasmic domain and was not observed in parental PC12 cells (Fig. 1*B*). In several cell lines that overexpressed the mR protein, wild-type p75 was decreased or absent. Western blot analysis of two independent clones, mR-1 and mR-2, demonstrated that these two lines displayed high levels of the truncated mR protein and nearly undetectable levels of endogenous p75 (Fig. 1*C*).

Analysis of NGF Receptors. The expression of p75 and $p140^{trk}$ was examined in further detail in mR-1 cells by affinity crosslinking with ^{125}I-NGF. Agents used to promote crosslinking exhibit differential effects upon the two receptors. In the presence of EDAC, ^{125}I-NGF is preferentially crosslinked to p75 (11). DSS is more efficient at crosslinking ^{125}I-NGF to $p140^{trk}$ (22).

Affinity crosslinking of PC12 and mR-1 cells with DSS resulted in species of apparent molecular mass 160 kDa, consisting of $p140^{trk}$ bound to ^{125}I-NGF. This crosslinked species was specifically recognized by antiserum to NGF and this recognition could be blocked with an excess of unlabeled NGF (Fig. 2*A*). No significant differences were observed in the levels of $p140^{trk}$. Crosslinking with EDAC indicated p75 was considerably lower in mR-1 cells (Fig. 2*B*), confirming the immunoblot analysis in Fig. 1*C*. As a comparison, PC12 and other mR-transfected cells (mR-3 and mR-6) displayed higher levels of p75 (Fig. 2*B*). Among these transfectants, there appeared to be an inverse relationship between level of

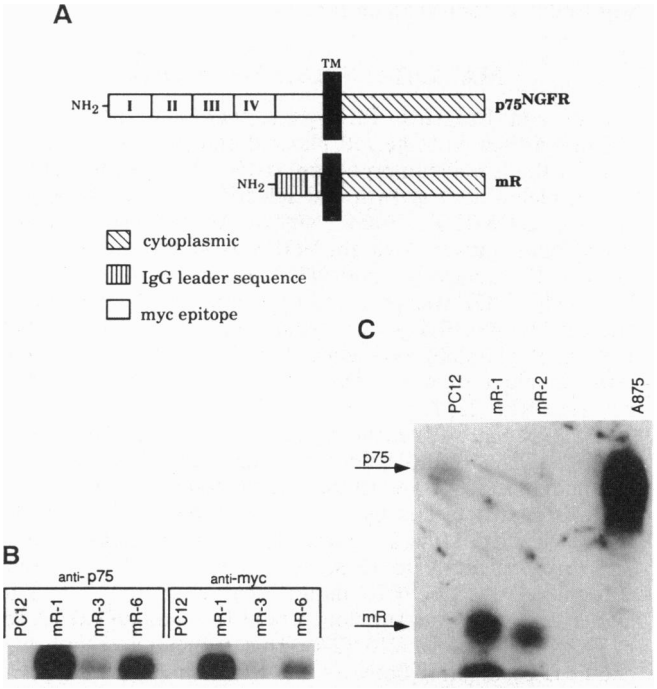

FIG. 1. (*A*) Schematic representation of the wild-type $p75^{NGFR}$ and the mR mutant. The extracellular domain of p75, consisting of four cysteine-rich repeats, was removed and replaced by the IgG leader sequence (vertical lines) followed by a Myc epitope sequence. The transmembrane (TM) and cytoplasmic (hatched) domains are identical in the wild-type and in the mutant. (*B*) Expression of the mR protein in transfected PC12 cells. Cell lysates were prepared and immunoprecipitated either with antiserum raised against p75 or with 9E10 ascites fluid (anti-Myc). Proteins were then electrophoretically transferred to nitrocellulose and probed with p75 antiserum. The mR protein appears with an apparent molecular mass of 28 kDa. (*C*) Western blot analysis of p75 and mR protein expression in PC12, mR-1, mR-2, and A875 cells. Equal amounts of protein were analyzed with polyclonal antibodies against the cytoplasmic domain of p75. Arrows designate the sizes of the mR protein and the full-length p75 protein.

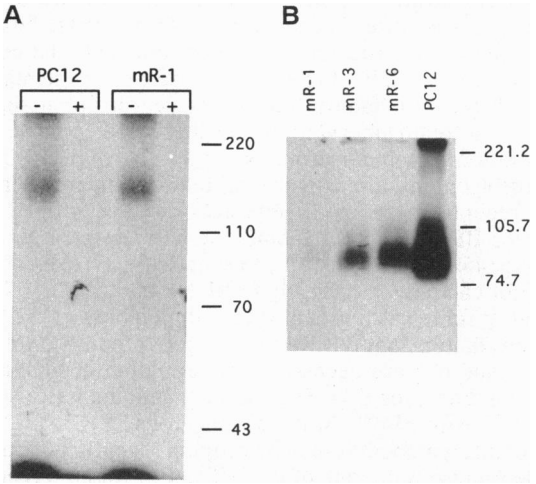

FIG. 2. ^{125}I-NGF affinity crosslinking. NGF was labeled with ^{125}I to high specific activity (3000 cpm/fmol) and used at a concentration of 10^{-10} M. (*A*) Affinity crosslinking to $p140^{trk}$ was performed with DSS on an equal number of PC12 cells and mR-1 cells (2×10^6 cells per ml). After crosslinking, the cells were lysed and crosslinked receptor complexes were immunoprecipitated with anti-NGF antiserum. An incubation with a 1000-fold excess of unlabeled NGF was also performed (+) to demonstrate specificity of the crosslinked product. (*B*) Affinity crosslinking on p75 was performed with cell suspensions from PC12 cells and from three independent clones of mR-transfected PC12 cells. An equal number of cells at the same concentration (2×10^6 cells per ml) were used. After crosslinking with 5 mM EDAC, the cells were lysed and the crosslinked products were immunoprecipitated with p75 antiserum. Molecular size markers (kDa) are indicated at right of each panel.

mutant p75 mR receptors and endogenous p75 receptor expression. Taken together, the data indicate that the PC12-derived mR-1 cells were deficient in p75.

The reasons for the decrease in full-length p75 receptor levels in mR-1 cells are unclear. Steady-state levels of p75 mRNA and Trk mRNA in mR transfectants were comparable to those in PC12 cells, as measured by Northern blot analysis (data not shown). Additional studies are needed to determine whether differential rates of turnover of p75, or decreased processing to the plasma membrane, or receptor internalization (30) is responsible for this effect. Overexpression of the mR protein may have affected biosynthesis or processing of endogenous p75. The change in receptor level observed here did not appear to be due simply to the transfection procedure, since PC12 cells transfected with many other cDNAs have not displayed such marked differences in the levels of NGF receptors.

Equilibrium Binding. PC12 cells display two classes of NGF binding sites, of high and low affinity (31, 32). A small, but significant, percentage (5–15%) of the sites constitute high-affinity ($K_d = 10^{-11}$ M) binding, and the remainder of the sites exhibit low-affinity binding ($K_d = 10^{-9}$ M). Whether $p140^{trk}$ and p75 together form high-affinity sites, or whether $p140^{trk}$ alone represents the high-affinity binding site, has been debated (7, 8, 11, 13, 33). If both p75 and $p140^{trk}$ participate in formation of high-affinity sites, then a shift in the level of either receptor would be expected to change the extent or the affinity of NGF binding.

Transfected mR-1 cells expressed Trk, but displayed decreased levels of wild-type p75. Equilibrium binding with ^{125}I-NGF on membrane preparations from mR-1 cells yielded a linear Scatchard plot (Fig. 3), with few detectable high-affinity sites. This analysis was carried out on four separate mR-1 preparations without any evidence for a curvilinear or biphasic binding curve. Indeed, at the lowest concentration of ^{125}I-NGF used in this analysis (1–6 pM), specific binding was not detected. Computer analysis of the binding data from mR-1 cells defined a single class of binding site, $K_d = 6 \times 10^{-10}$ M, whose affinity for NGF is substantially lower than that of the high-affinity site. A similar binding profile was measured for mR-2, an independent clone like mR-1 (Fig. 1*C*). Thus, these binding results were not a result of clone-to-clone variation. In contrast, NGF binding to native, wild-type PC12 cell membranes produced two distinct equilibrium binding sites (Fig. 3), with K_d values of 3.5×10^{-11} M and 4.9×10^{-9} M. High-affinity binding in mR-1 was greatly reduced, in comparison to PC12 membranes, which produced 3000 sites per cell.

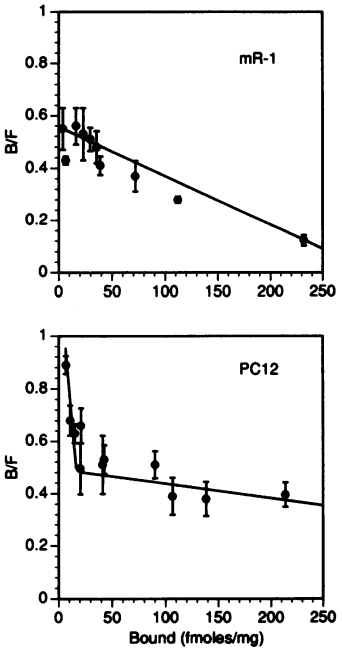

FIG. 3. Equilibrium binding of ^{125}I-NGF to PC12 and mR-1 cells. Membranes were prepared and assessed for binding at 30°C (11). Nonspecific binding was measured in identical binding reactions with the exception that an excess of unlabeled NGF was included. All binding reactions were analyzed by the LIGAND computer program (11). B/F, bound/free.

Since Trk levels were not drastically altered in mR-1 cells, these results indicate that the reduction in p75 receptors is responsible for the decrease in the number of high-affinity binding sites. It is also possible that overexpression of truncated p75 receptors may have contributed to the lack of high-affinity binding, possibly by disrupting potential interactions between the two receptors. That the p75 cytoplasmic domain may influence NGF binding is supported by deletion studies of p75 binding in the presence of $p140^{trk}$ (23, 29). Taken together, these data establish that high-affinity NGF binding can be compromised by altered expression of p75.

Neurite Outgrowth. To investigate whether biological responsiveness is influenced by the noted changes in p75 levels and steady-state binding, neurite outgrowth in response to NGF was examined. mR-1 cells displayed pronounced process outgrowth (Fig. 4) at concentrations of NGF from 1 to 100 ng/ml, similar to NGF-treated PC12 cells (Table 1). Interestingly, mR-1 cells displayed a higher background of neurite outgrowth, in the absence of neurotrophin. The mR-1 cells have a substantially different ratio of full-length p75 to $p140^{trk}$, which probably leads to a relative lack of high-affinity binding sites. The ability of these cells to respond to NGF implies that some biological responses may be uncoupled from high-affinity binding.

Responsiveness to NT-3. Neurotrophins can interact with multiple Trk receptors (14, 34, 35). Indeed, $p140^{trk}$ (TrkA) is stimulated not only by NGF but also by NT-3, NT-4, and NT-5 (8). It must be noted that the majority of these findings have been obtained in transfected fibroblasts. In a neuronal cell context, responsiveness through activation of $p140^{trk}$ is likely to be more restricted (33, 35).

We have examined the ability of NT-3 to cause differentiation in PC12 cells. Normally, NT-3 has a very limited or negligible effect upon PC12 differentiation (35, 36). This behavior was confirmed (Fig. 4 and Table 1). However, when mR-1 cells were treated with recombinant NT-3, a striking increase in the number of cells displaying neurite outgrowth was observed. In dose–response experiments, mR-1 cells clearly responded at a range of NT-3 concentrations that did

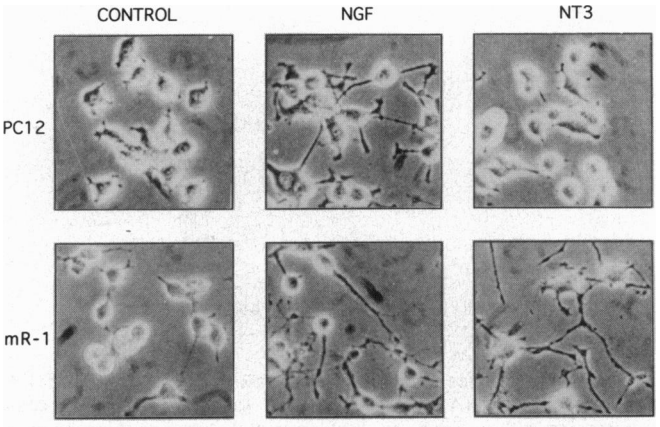

FIG. 4. Differentiation of mR-1 and PC12 cells following treatment with NGF and NT-3. Cells were treated for 2 days with mouse NGF (1 ng/ml) or 1 recombinant human NT-3 (1 ng/ml).

Table 1. Comparative responses of PC12 and mR-1 cells to NGF and NT-3

Conc., ng/ml	% neurite-containing cells			
	PC12		mR-1	
	NGF	NT-3	NGF	NT-3
0	2.7 ± 2.5	1.1 ± 0.4	12.9 ± 4.3	10.3 ± 1.0
0.1	4.8 ± 1.8	0.9 ± 0.4	14.7 ± 3.4	10.4 ± 3.0
1.0	30.0 ± 2.0	1.0 ± 1.0	33.1 ± 2.5	15.3 ± 6.4
10	63.9 ± 9.1	1.5 ± 0.7	64.5 ± 3.0	45.1 ± 15.0
100	83.7 ± 11.0	1.8 ± 0.4	72.2 ± 4.5	65.6 ± 3.8

Equal numbers of cells were treated in 60-mm dishes for 3 days in the presence of NGF (Bioproducts for Science) or NT-3 (Chiron) at 0–100 ng/ml. Cells exhibiting neurites of >2 cell-body diameters were counted in random fields containing 200–300 cells.

not affect the parental PC12 cells (Table 1). Another cell line, mR-2, expressing few full-length p75 receptor molecules (Fig. 1*C*), also responded to NT-3 at similar concentrations (data not shown), indicating that this response was not due to clonal variation.

Differentiation of mR-1 cells by NT-3 could occur by activation of TrkA, TrkB, or TrkC (8). Neither TrkB nor TrkC, the bona fide receptor of NT-3 (4), is expressed in PC12 cells (35, 36). In agreement, RNA blot analysis indicated only TrkA mRNA, and not TrkB and TrkC mRNA, in the PC12-derived mR-1 cells (Fig. 5). Therefore, TrkA serves as the receptor for NT-3 in these cells. Decreased expression of p75 in mR-1 cells may have contributed to the ability of NT-3 to elicit a response. Consistent with this assessment is the virtual absence of NT-3 induced neurite outgrowth in other lines, mR-3 and mR-6 (Fig. 6), which expressed higher levels of p75 than mR-1 (Fig. 2*B*). Effects of NT-3 and other neurotrophins have also been observed in fibroblasts expressing only p140^{trk}, in the absence of p75 (14, 34). Thus, a crucial role for p75 expression may be to provide more discrimination, by allowing only NGF to act and restricting the action of other neurotrophins, such as NT-3.

DISCUSSION

Growth factors are known to signal through multiple receptor subunits, as exemplified by the cytokine family including interleukins 2, 5, and 6 and granulocyte/macrophage-colony-stimulating factor (37), and by the transforming growth factor β family (38). For NGF, ligand–receptor interactions in responsive cells involve 140^{trk} and p75. The Trk NGF receptor is capable of signaling by a mechanism similar to other receptor tyrosine kinases (39).

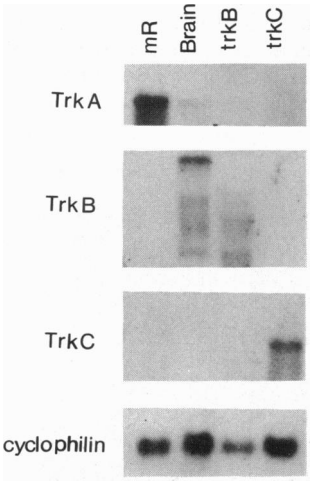

FIG. 5. Transfected PC12 cells (mR-1) express only TrkA mRNA. Total RNA from mR-1 cells, rat brain, and 3T3 cells expressing either TrkB or TrkC receptor tyrosine kinases was isolated, electrophoretically separated, and hybridized in quadruplicate blots with probes specific for TrkA, TrkB, or TrkC (36) or for cyclophilin (44).

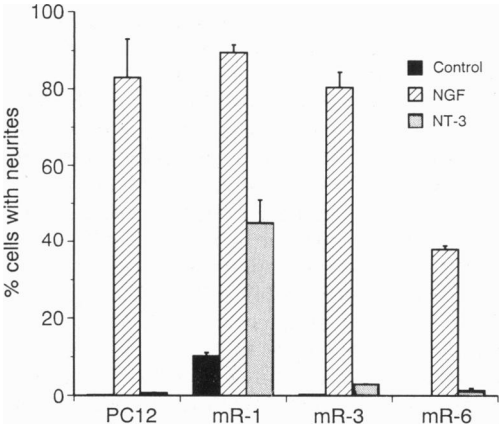

FIG. 6. Neurite outgrowth in PC12 and mR-transfectant cells treated with NGF and NT-3. The percentage of cells giving rise to neurites of >2 cell diameters was counted in five random groups of 300–400 cells. Cells were treated for 2 days in the presence of NGF (10 ng/ml) or NT-3 (10 ng/ml). The mean and standard error are presented.

The p75 neurotrophin receptor is a member of a family which includes two receptors for tumor necrosis factor, CD30, CD40, the Fas antigen, and OX40 (40). The cytoplasmic domain of each family member is distinctive, without a discernible enzymatic domain, but may serve signaling functions. For p75, the transmembrane and cytoplasmic domains are highly conserved between species, even more so than the extracellular binding domain (41), suggesting a functional role for these domains in neurotrophin signaling.

Binding experiments of NGF with p140^{trk} in the presence of mutant p75 receptors lacking a cytoplasmic domain yielded only low-affinity binding, implying that an intact p75 receptor is required to form a high-affinity complex (23, 29) and that p75 does not function merely as a presentation receptor. Since p75 binds to all the neurotrophins (9, 10), it has also been suggested that p75 enhances discrimination of neurotrophin factors, possibly by forming receptor complexes with Trk family members (7).

The results here suggest that the lower levels of p75 in the mR-1 cell line contribute to the observed decrease in high-affinity sites. The p75 receptor is found at higher levels than p140^{trk} in NGF-responsive cells. These observations are supported by experiments in which an excess of p75 over p140^{trk} is required for high-affinity binding (11), and by p75-antibody blocking experiments that reduced high-affinity binding (16). A requirement for a ratio of appropriate receptor numbers may also explain the inability to observe an increase in high-affinity sites in other studies (13). Formation of high-affinity NGF binding sites is dependent upon the level of expression and the stoichiometry of both receptors.

An important observation is the finding that mR-1 cells respond morphologically to NGF at 1–100 ng/ml (10^{-11}–10^{-9} M), even though the K_d of binding to p140^{trk} is $\approx 6 \times 10^{-10}$ M. These results imply that only a small percentage of receptor occupancy is necessary for biological action. This property has been documented for the effects of NGF, BDNF, and NT-3 on chicken embryonic sensory neurons, wherein the half-maximal concentration necessary for biological effects is at least an order of magnitude below the K_d of high-affinity binding (33).

The results in this study also demonstrate that signaling by NGF receptors and the ability to form high-affinity sites may be separable events. This conclusion is contrary to the conventional dogma, which assumes that high-affinity binding is required for neuronal survival and differentiation. Biological responses may be obtained without benefit of intact high-affinity binding conditions. For example, reconstitution of high-affinity sites after transfection of NGF receptors has yielded cells displaying limited signaling, such as increased neurite outgrowth (42). High-affinity binding can be therefore potentially dissociated from neurotrophin responsiveness.

We have shown that a switch in neurotrophin responsiveness occurs in PC12 cells in which high-affinity binding has been altered and the ratio of p75 to $p140^{trk}$ has been significantly decreased. NT-3 is fully capable of inducing neurite formation in mR-1 cells deficient in p75 but does not elicit neurite outgrowth from PC12 cells (Fig. 4). These results are compatible with a model in which coexpression of p75 and $p140^{trk}$ permits NGF to signal more selectively over other neurotrophins, in the presence of limiting concentrations of factors. This model implies that expression of $p140^{trk}$ alone can lead to signaling, but without the ability to form many high-affinity sites, or respond with exclusive specificity.

An important role of p75 may be to provide more specificity to the interaction of neurotrophins with Trk family members. The expression of high levels of p75 appears to be an important factor in restricting the ability of $p140^{trk}$ to respond to NGF, and not NT-3. This differential behavior is reflected in the binding of NT-3 to p75, which occurs with much slower kinetics than NGF binding to p75 (10). This discriminatory role for $p75^{NGFR}$ may also provide an explanation for how TrkA in p75 mutant mice (18) could potentially respond to other neurotrophin factors. A prediction from this study is that other Trk receptor family members may respond far more selectively to different neurotrophins when the p75 neurotrophin receptor is expressed. An alternative model concerning the cell lines described here is that the cytoplasmic domain of p75 may have disrupted normal p75–p140 interactions and allowed NT-3 to become more accessible to $p140^{trk}$. Expression of the truncated receptor may confer activities of the native receptor, as evidenced by the higher level of neurite outgrowth in the absence of neurotrophins. The p75 receptor may possess a signaling capability, since biological responses have been noted in experiments in which epidermal growth factor receptor/$p75^{NGFR}$ chimeras were expressed in PC12 cells (43).

Interactions between neurotrophin receptors have yet to be fully elucidated, but the requirement for a ratio of p75 and $p140^{trk}$ for high-affinity site formation implies that receptor oligomerization may take place. Definition of the interactions that exist between these two classes of neurotrophin receptors will ultimately reveal how specificity in neurotrophin action is achieved.

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