The Cytoplasmic and Transmembrane Domains of the p75 and Trk A Receptors Regulate High Affinity Binding to NGF

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Abbreviations: NGF, nerve growth factor, PBS, phosphate buffered saline, RIPA, radioimmunoprecipitation assay, m.o.i., multiplicity of infection

Running title: NGF high affinity binding site formation
Abstract

Ligand-induced receptor oligomerization is an established mechanism for receptor tyrosine kinase activation. However, numerous receptor tyrosine kinases are expressed in multicomponent complexes with other receptors that may signal independently or alter the binding characteristics of the receptor tyrosine kinase. NGF interacts with two structurally unrelated receptors, the Trk A receptor tyrosine kinase and p75, a TNF receptor family member. Each receptor binds independently to NGF with predominantly low affinity ($K_d=10^{-9} \text{M}$), but produce high affinity binding sites ($K_d=10^{-11} \text{M}$) upon receptor co-expression. Here we provide evidence that the number of high affinity sites is regulated by the ratio of the two receptors, and by specific domains of Trk A and p75. Co-expression of Trk A containing mutant transmembrane or cytoplasmic domains with p75 yielded reduced numbers of high affinity binding sites. Similarly, co-expression of mutant p75 containing altered transmembrane and cytoplasmic domains with Trk A also resulted in predominantly low affinity binding sites. Surprisingly, extracellular domain mutations of p75 that abolished NGF binding still generated high affinity binding with Trk A. These results indicate that the transmembrane and cytoplasmic domains of Trk A and p75 are responsible for high affinity site formation and suggest that p75 alters the conformation of Trk A to generate high affinity NGF binding.
Introduction

Growth factor receptor tyrosine kinases, such as the epidermal growth factor receptor (EGFR) undergo ligand-induced oligomerization, leading to receptor activation. Although homodimerization of receptor tyrosine kinases is sufficient to initiate transmembrane signaling, an increasing number of receptor tyrosine kinases have been found to be co-expressed with heterologous co-receptors lacking extrinsic kinase activity. Examples of such heteromeric receptors include the receptors for vascular endothelial growth factor (VEGF), the flt-1 receptor tyrosine kinase and neuropilin-1 (1); the receptors for the glial derived neurotrophic growth factor (GDNF), the ret receptor tyrosine kinase and GRFα (2) and the receptors for nerve growth factor (NGF), the Trk A receptor tyrosine kinase and the p75 neurotrophin receptor (3). With the VEGF and NGF ligands, co-expression of their receptor tyrosine kinase and heterologous co-receptor generates receptor complexes which exhibit higher affinity binding constants than those exhibited by homodimeric receptor tyrosine kinase complexes. For GDNF receptor activation, expression of both the ret receptor tyrosine kinase and GRFα subunit is required for ligand binding, as GDNF is unable to activate homodimeric ret receptor complexes (4).

Several models have been proposed to accommodate co-operative roles for these dual receptor systems. A model in which the non-receptor tyrosine kinase first binds ligand, alters the local concentration, and then passes the ligand, to the receptor tyrosine kinase has been proposed. A second, conformational model, predicts that co-expression of both receptor tyrosine kinase and co-receptor alters the conformation of the receptor tyrosine kinase through allosteric interactions, generating a higher affinity binding site by altering of the association or dissociation constants of the ligand with its receptor tyrosine kinase.

We have utilized the NGF receptors as a model system to clarify mechanism that generates high affinity site formation. NGF belongs to the neurotrophin family of survival and differentiation factors which also includes BDNF, NT-3 and NT-4/5. NGF responsive neurons exhibit two classes of binding sites, based upon equilibrium binding reactions (5). The transmembrane proteins responsible for high affinity NGF binding (Kd 10^{-11} M) are the Trk A receptor and the p75 neurotrophin receptor (which binds all neurotrophins (6)). Kinetic analysis of NGF binding indicates that each receptor binds NGF with a relatively low affinity K_d between 10^{-9} and 10^{-10} M (7). While p75 displays very rapid rates of association and dissociation with NGF, the Trk A receptor has much slower on- and off-rates. When Trk A receptors are co-expressed
with p75, the rate of association is accelerated 25 fold, generating a new kinetic site exhibiting high affinity binding properties (7). Thus, one function of p75 is to increase the binding affinity of NGF. How this is accomplished has not been determined.

Direct interactions between the p75 and Trk A receptors have been difficult to document biochemically, although ligand induced receptor homodimers can be readily detected in affinity crosslinking reactions (8). However, immunoprecipitation experiments using $^{125}$I crosslinked to neural tissue or cell lines suggests that an association between the Trk A and p75 may take place (9,10,11). Co-precpitation of endogenous Trk A and p75 in PC12 cells indicates the receptor complex may form without NGF (12). Photobleaching recovery experiments using fluorescently tagged p75 receptors, or using monovalent antibody detection of Trk A have also revealed physical clustering of p75 with Trk A mediated by both intracellular and extracellular domains of p75 and Trk A, and augmented by Trk A kinase activity (13). These studies have recently been extended to other Trk family members, with the demonstration of a Trk B and p75 interaction by co-immunoprecipitation mediated by intracellular and extracellular domains (14). Furthermore, co-expression of p75 with Trk B was found to modulate the ligand specificity of Trk B (14).

In addition to altering ligand binding, co-expression of p75 with Trk A can also influence Trk A signaling (15-18). Binding of NGF to Trk A receptors activates its cytoplasmic kinase, resulting in the phosphorylation of cytoplasmic tyrosine residues, followed by the binding and activation of multiple proteins including the Shc, PLCγ, FRS-2 and SH2B (19-21) which mediate effects such as survival and differentiation. The p75 receptor is a member of the TNF family of receptors, and contains a putative cytoplasmic death domain (22). Although the p75 receptor can modulate Trk activity, and alter the specificity of Trk receptors for neurotrophin ligands (14,16,23), the p75 receptor can also mediate cell death when expressed independent of Trk receptors (24-26). Neurotrophin binding to p75 can result in sphingomyelinase activation (27) and recruitment of TRAF-6, a mediator of TNFR activation (28), RhoA (29), and NRIF (30). Thus receptor-mediated signal transduction by the neurotrophins is unique among polypeptide growth factors as two different transmembrane signaling receptors can by activated by a neurotrophin ligand, with distinctive biological outcomes. In addition, the ability of each receptor subunit to modulate the signaling cascades initiated by the co-receptor suggests that the receptors directly interact.

One potential mechanism for the p75 receptor’s ability to influence high affinity binding may be to increase the effective concentration of neurotrophin at the cell surface.
and thus enhance NGF binding to Trk A (16). A similar mechanism involving heparin
sulfate facilitation of FGF binding to FGFR2 has been postulated (31), but a more
complex role in inducing conformational changes in the FGFR2 complex has become
apparent with crystallographic analysis (32). Another mechanism for p75 regulation of
high affinity site formation is that the conformation of Trk A may be altered in the
presence of p75, and this allosteric regulation facilitates Trk A: ligand binding and
subsequent signaling functions (7,10). To test these hypotheses that we undertook
structure/function analysis of the p75 and Trk A receptors in order to identify the
domains of each receptor required for high affinity ligand binding.

Materials and Methods
Baculovirus Vectors: The generation of the baculovirus constructs containing cDNAs
for wild type and mutant p75 and Trk A have been described (33), as have the
generation of chimeric cDNAs encoding Trk A:torso constructs (34). The cDNAs
encoding the epidermal growth factor receptor: p75 chimeras (constructs EN10 and
EN31) (35) were released by EcoRI digestion from the Bluescript vector, and ligated into
the Nco to EcoRI site of the baculovirus expression vector using PCR primers.

Mammalian expression vectors: The cDNA for the mutant p75 construct (105) (36) was
excised from Bluescript using EcoRI digestion, and subcloned into the pCMV
expression vector. The construction of pCMV5 plasmids encoding the native Trk A and
p75, and a Trk A:Trk B chimeric receptor (3.2B) have been described (37). This 3.2B Trk
A:Trk B chimera contains Trk A sequence in the IgGC1 and C2 domains, with the
leucine rich repeats, transmembrane and intracellular domains of Trk B origin. A
second Trk A: Trk B chimeric receptor, containing the Trk A IgGC1 and C2 domains,
and the Trk A transmembrane domain, with Trk B leucine rich repeats and intracellular
domain, was constructed by domain swapping using the PCR primers and techniques
described in detail (37). The nucleotide sequence of each cloned fragment from Trk A
and Trk B was determined using the dideoxy chain termination method.

Cell lines: Sf9 insect cells were maintained in TMN-FH medium from JRH Biosciences
supplemented with 9% heat inactivated fetal bovine serum and 50 µg/ml gentamycin at
28°C. PC12 nmr5 cells expressing mutant Trk A receptors were cultured as described
(33). Human embryonic kidney cell line 293 was maintained as described (35).

Baculovirus Expression in Sf9 cells: To express a single NGF receptor, recombinant
baculovirus as added to Sf9 cells (2 X 10^6 cells in a 25 cm^2 flask) at a multiplicity of infection of 1 for the p75 virus, or 4 for the Trk A virus. For co-expression experiments, baculovirus encoding Trk A was added to SF9 cells at a moi of 16 followed immediately by the addition of virus encoding p75 at a m.o.i. of 1. To vary the ratio of infection, the m.o.i. of virus encoding Trk A was varied from 2 to 40 and the m.o.i. of virus encoding p75 was varied from 1 to 10. After 60 hours of infection, cells were pelleted by centrifugation, washed in phosphate buffered saline and snap frozen in liquid nitrogen.

Expression in mammalian cells. 293 cells were cotransfected with the pCMV p75 or pCMV mutant p75 (105) construct and the pMEX construct, which encodes a neomycin resistance gene, using the calcium phosphate precipitation method (37). Following selection in media containing 250 µg of G418, colonies were subcloned and expanded. The level of expression of native or mutant p75 was determined using whole cell lysates in Western blot analysis with the 9992 antisera specific for the intracellular domain of human p75 (36). Clones expressing native or mutant p75 were subsequently transiently transfected with pCMV5 encoding Trk A, using the calcium phosphate method, and incubation of the cells with DNA for 4 hours, followed by replacement with fresh media. Twenty eight hours after addition of DNA, cells were harvested in phosphate buffered saline containing 1mM EDTA. For membrane binding assays, cells were pelleted and snap frozen in liquid nitrogen. For binding analysis using intact cells, cell suspensions were utilized immediately upon harvesting.

Western blot analysis. Following lysis of cells in RIPA, protein content was determined by the Biorad assay using bovine serum albumin as a standard (38). Equivalent concentrations of protein were subjected to separation by SDS-PAGE, blotted onto nitrocellulose, and probed with the 9992 antisera to intracellular epitopes of p75 (7), anti-Trk antisera 203 detecting intracellular epitopes (3), Trk A-Out detecting extracellular epitopes (39) or antisera to torso (34) as described. Immunocomplexes were detected by enhanced chemiluminescence technique (Amersham), and subjected to densitometry.

Equilibrium binding assay using radioiodinated NGF. Mouse NGF (Bioproducts for Science, renin free), was radioiodinated using lactoperoxidase and hydrogen peroxide as described (38). The specific activity averaged 3000 dpm/fmol, and was used within two weeks of radioiodination. Crude membrane preparations from snap frozen cells
were prepared as described (7). The equilibrium binding assay conditions utilizing membrane preparations have been described in detail (7,38), utilizing 125I-NGF concentrations from 0.0005 to 4nM and each condition was assessed in triplicate, in the presence or absence of excess unlabeled (0.8 µM) NGF. The specific counts averaged 60-85% of the total counts. In indicated experiments, equilibrium binding studies were performed using the whole cells binding assay (7). Cells were resuspended at 0.75 X 10^6/ml final concentration, and binding to radioiodinated NGF (0.0005 to 4 nM), in the presence or absence of excess unlabeled NGF (0.8 µM) proceeded for 2h at 0.4°C. Cell bound NGF was separated from free NGF by pelleting through calf serum. The Scatchard plot analysis was performed using the LIGAND program as described (7), and additionally analyzed using the PRISM program, to perform nonlinear regression, and directly compare curve fits to determine if they were similar with 95% confidence limits.

Results
The ratio of expression of p75 and Trk A regulates the percentage of high affinity binding sites.

The level of expression of the p75 and Trk A receptors are highly regulated during neuronal development, and in response to injury. To determine the optimal levels of expression of each receptor which confers the maximal percentage of high affinity binding sites, binding studies were performed using cells which expressed varying ratios of Trk A:p75. In prior studies using PC12 cells, we had noted that expression of Trk A:p75 at ratios of 1.0:0.8 yielded a higher percentage of high affinity sites as compared to PC12 cells with a 1:20 ratio of Trk A:p75 (42). Transient expression of Trk A and p75 in Sf9 cells using baculovirus expression vectors allowed the two receptors to be expressed at defined ratios by altering the ratio of infectious virus, and generated Sf9 cells which expressed high levels of receptors necessary for equilibrium binding studies. The level of expression of each receptor in infected Sf9 cells was determined by Western blot analysis (Fig. 1A) in comparison with detergent extracts of Trk A overexpressing PC12 (Trk A - PC12) cells, which express 70,000 Trk A and 90,000 p75 receptors per cell, as assessed by kinetic analysis of NGF binding sites in whole cell preparations (7) (Fig. 1B).

Expression of the Trk A receptor at levels significantly in excess of p75 results in predominantly low affinity binding sites. At ratios of Trk A: p75 of approximately 20:1 only low affinity binding sites were detected, and exhibited a K_d of 1.1 X 10^-9M (Fig. 1E
and Tables 1 and 2). Conversely, when p75 was expressed at levels significantly in excess of Trk A (ratio of Trk A:p75 of 1:15) only 3% of the sites exhibited high affinity binding ($K_d = 1.6 \times 10^{-11}M$) (Fig. 1B and Tables 1 and 2). However, as the ratio of receptors became closer to equivalency, (ratios of Trk: p75 of 1:5 or 1:0.8) the percentage of high affinity sites ($K_d = 2.4-2.8 \times 10^{-11}M$) increased to 7% or 10% of the total binding sites, respectively (Fig. 1C,D and Tables 1 and 2). Indeed, the percentage of high affinity sites and the $K_d$ of the high affinity site observed in Sf9 cells expressing Trk A:p75 at a 1:0.8 ratio is comparable to that observed using Trk A overexpressing PC12 cells at a 0.8:1 ratio (7,42), demonstrating that binding results obtained with whole cells and membrane preparations are consistent, and that receptor expression in different cell types yields binding sites with similar affinity (7,3,42). Thus, the ratio of expression of Trk A to p75 can significantly alter the number of high affinity sites, with a higher percentage of the total receptors exhibiting high affinity binding when the receptors are expressed in near equimolar ratios.

The transmembrane and cytoplasmic domains of Trk A affect high affinity site formation.

The region of the extracellular domain of Trk A that mediates binding to NGF has been mapped to the juxtamembrane IgG C2 domains (40,41). However, the effects of alterations within the transmembrane and cytoplasmic domains upon NGF binding are unknown. To assess the role of each of these domains, the transmembrane and cytoplasmic domains of the Torso receptor kinase were exchanged with the Trk A receptor. Torso is a distantly related receptor tyrosine kinase to Trk A, and a series of chimeric Trk A: Torso receptors were generated (34) to test the contribution of each domain within the context of the full length receptor tyrosine kinase (Fig. 2A). These constructs were co-expressed with p75 at a Trk:p75 ratio of approximately 1:1 (Fig 2B). This ratio of expression results in 13% high affinity site formation sites ($K_d = 1.7 \times 10^{-11}M$ and Tables 1 and 2), expressed as a percentage of total binding, upon co-expression of native Trk and p75 (Fig. 2C). Both the transmembrane and cytoplasmic domains of Trk A appear to contribute to high affinity site formation, when coexpressed with p75, as chimeric proteins with torso sequences substituted for either of these two Trk A domains displayed reduced percentages of high affinity sites, to approximately 4-6% of the total binding sites (305:p75, 4% binding with $K_d = 1.8 \times 10^{-11}M$; 303:p75, 6% binding with $K_d = 7 \times 10^{-11}M$)(Fig 2D and 2E and Tables 1 and 2). Chimeric receptor, encoding the Trk A extracellular sequence and the torso transmembrane and
cytoplasmic domains similarly yield a reduced percentage of high affinity sites upon coexpression with p75 (325:p75, 4% binding with $K_d = 4 \times 10^{-11}$M, Fig 2F and Tables 1 and 2). Although the generation of chimeric receptors from structurally distinct proteins can alter protein conformation, these results suggest that the cytoplasmic and transmembrane domains of Trk A can influence the ability of the extracellular domain to bind NGF with high affinity at equilibrium.

To more precisely map the domains of Trk A which are necessary to confer high affinity binding when co-expressed with p75, a series of chimeric Trk A: Trk B receptors were utilized. These highly related receptors, which share 70-85% identity across all domains, nonetheless discriminate between the related neurotrophins NGF and BDNF. Although the IgG C2 domain of Trk A is sufficient to confer NGF binding to Trk A (37, 40), we sought to assess the roles of the transmembrane and cytoplasmic domains of the Trks in modulating high affinity NGF binding when coexpressed with p75. A chimeric Trk A: Trk B receptor, generated by swapping Trk A sequence in the IgG C2 domains on a Trk B backbone (Fig. 3A, 3.2), and a chimera swapping both the Trk A IgG C domains and the Trk A transmembrane domain for Trk B sequence (3.3) were utilized in co-expression studies. Cells expressing Trk chimeras: p75 at ratios of approximately 1:3 were generated for equilibrium binding studies (Fig 3B). Although Trk A IgG C2 domains are sufficient to confer low affinity NGF binding, co-expression of a Trk chimera of Trk A IgG C plus Trk A transmembrane domains, with native p75, yields high affinity sites (14% binding with $K_d = 2.6 \times 10^{-11}$M) (Fig 3C and Tables 1 and 2). These results are comparable to those obtained upon coexpression of TrkA and p75 in 293 cells (Fig 3E, 12% binding with $K_d = 2.3 \times 10^{-11}$M). These results suggest that the highly related intracellular domain of Trk B (87% identical to Trk A) can effectively substitute for the intracellular domain of Trk A in terms of high affinity site formation as the $K_d$ of the high affinity site is similar to that obtained with native Trk A co-expression with p75 (compare to Fig. 1D and 2C). In contrast, co-expression of a Trk chimera of Trk A IgG C plus Trk B transmembrane domain, with native p75, yields only intermediate and low affinity binding sites ($K_d = 1.8 \times 10^{-10}$ M and $K_d = 0.9 \times 10^{-9}$ M, respectively) (Fig 3D and Tables 1 and 2). Thus, both the IgG C2 and transmembrane domains of Trk A are required for the generation of high affinity binding sites (Fig. 3C,D, and Tables 1 and 2).

To further assess the contributions of the cytoplasmic domain of Trk A, a point mutation, K538A, which renders Trk A kinase inactive (33) was expressed by transfection of PC12 nnr5 cells expressing endogenous p75. In the PC12 nnr5 cells, the ratio of Trk A(K538A) to p75 was approximately 1:0.7 (Fig 4A), however, high affinity
binding sites (Kd < 1 × 10^{-10} M) were not generated (Fig 4B and Table 1). Thus, not only is the cytoplasmic domain of Trk A required for high affinity site generation, but kinase activation contributes to the formation of this site (Tables 1 and 2).

**Domains of p75 which contribute to high affinity site formation.**

The extracellular domain of p75 interacts with NGF throughout the four cysteine rich sequences, although the most N-terminal sequence appears dispensable for low affinity binding (36,43). In prior studies, we have demonstrated that the cytoplasmic domain of p75 is required for high affinity site formation, since expression of a truncated p75 encoding only 8 amino acids within the cytoplasmic domain in cells which express Trk A results in only low affinity binding sites (38).

To assess whether p75 extracellular sequences contribute to high affinity binding, chimeric p75 receptors were generated, exchanging the extracellular domain of the EGF receptor for the extracellular domains of p75, EGFR-p75(10) (Fig. 5A)(35). This chimeric receptor, which lacks the extracellular domain of p75, does not bind NGF (35). Coexpression of this chimeric EGFR-p75 with native Trk A in SF9 cells at a ratio of approximately 1:1 (Trk A: chimeric EGFR /p75 (10), Fig. 5B) resulted in both high and low affinity sites. Approximately 14% of the sites exhibited a Kd = 1.9 × 10^{-11} M, with the majority of low affinity (Kd = 1.3 × 10^{-9} M) (Fig. 5C and Tables 1 and 2). These results suggest that the binding of NGF to p75 is dispensable for high affinity site formation. However, both the intracellular and transmembrane domains of p75 promote high affinity site formation. To assess whether the transmembrane domain of p75 contributes to high affinity site formation, a second chimeric EGF receptor:p75 receptor was utilized which contained both extracellular and transmembrane sequences from the EGF receptor, and the cytoplasmic domain of p75 (31 construct, Fig. 5A).

When this construct (31) was co-expressed with Trk A at a ratio of approximately 1:1 (Trk: chimeric EGFR/p75 (31), Fig. 5B), no high affinity sites were detectable (Kd = 1.6 × 10^{-9} M and Tables 1 and 2)(Fig. 5D). These studies suggest that upon co-expression with Trk A, the extracellular domain of p75 is dispensable for high affinity site formation, whereas the transmembrane domain of p75 is required.

To confirm that binding of NGF to p75 is not required for high affinity site formation, a mutant p75 receptor which is incapable of binding to NGF due to a five amino acid deletion in the third cysteine repeat was utilized in coexpression studies with Trk A (Fig 6A) (36). The inability of this mutant p75 to bind NGF was confirmed in crosslinking experiments utilizing radioiodinated NGF. Although a 90 kD crosslinked product is detectable in PC12 cells bearing native p75 receptors (13 kD for
NGF and 75 kD for p75), no product is detectable in cells expressing the mutant p75 (50) (Fig 6C), consistent with earlier studies (36). However, upon co-expression of the mutant p75 (50) with Trk A, at ratios comparable to those in Trk-PC-12 cells (or approximately 1:1, Trk: p75, fig. 6B), both high and low affinity sites for NGF were generated (8% of sites with $K_d = 2.8 \times 10^{-11}$ M, 92% of sites with $K_d = 1.4 \times 10^{-9}$ M)(Fig. 6D and Tables 1 and 2). In contrast, cells expressing Trk A alone yielded only low affinity NGF binding sites ($K_d = 1.5 \times 10^{-9}$ M) (Fig. 6D).

Collectively, these results suggest that the extracellular domain of p75 is not required for high affinity site formation (Tables 1 and 2). Strikingly, these chimeric receptor data support a model in which both the transmembrane and cytoplasmic domains of p75 are required for high affinity binding, most probably through a conformational change in the Trk A receptor.

Discussion

NGF responsive neurons of the peripheral nervous system require a continuous supply of subpicomolar concentrations of this neurotrophin for survival and maintenance, yet during development, exhibit critical periods in which sensitivity and requirements for NGF are distinctive from those exhibited by postnatal animals (44). These observations suggest that the ability of neurons to respond to neurotrophins can be exquisitely modulated, in part by regulating downstream signaling events, but also by the regulation of receptors on their cell surface. Our findings that the number of high affinity sites can be altered, simply by changing the ratio of expression of the two NGF receptor subunits, indicates that this receptor system imparts a cell with the ability to rapidly modulate cell responsiveness to limiting concentrations of ligand. In this regard, these studies are in agreement with those examining the survival of sensory neurons, in animals that are haploinsufficient for p75 (45), where lower levels of expression of the p75 receptor result in a survival disadvantage, in a gene dose dependent manner. These observations also imply that the massive upregulation of both Trk A and p75 noted following injury may function not only to increase the total number of binding sites but may also regulate the generation of binding sites with higher affinity, thus augmenting Trk A signaling and NGF internalization in response to stress.

Two hypothetical models of potential p75-Trk A interactions have been proposed, to accommodate cooperative roles of the two receptors (46). The presentation or sequestration model postulates that NGF first binds rapidly to p75, increasing the local concentration of NGF for Trk A. This model does not require a direct interaction
of p75 with Trk A receptors, and is supported by two classes of experiments: ligand mutagenesis studies, in which neurotrophins deficient in p75 binding exhibit Trk activation that is modestly impaired (47) and studies in which ligand blocking antibodies to p75 reduce Trk activation (48). A conformational model invokes an interaction of Trk A with p75 in the absence of ligand. This conformational model is supported by photobleaching recovery experiments, demonstrating preformed complexes of Trk A and p75 which can be detected in naive cells, but which will undergo further aggregation in the presence of exogenous ligand. Evidence for this interactive model has also been provided in co-immunoprecipitation studies, documenting p75: Trk A complexes (9,49) and p75:TrkB complexes (14). In the conformational model, co-expression of both receptors could result in a conformational change in Trk A, leading to an accelerated rate of association and thus, a high affinity binding site. This latter model is consistent with the data presented here. It should be emphasized, however, that neither of these models accurately depicts the exact stoichiometry of p75:Trk A but both models are consistent with dimerization of Trk following binding of ligand.

The structural: functional relationship of the domains of Trk A and p75 which are required for high affinity binding suggest that interactions between the two receptors occur via transmembrane and cytoplasmic domains. These observations are consistent in part with those of Ross and colleagues (38), who have determined that deletions in the cytoplasmic domain of p75, or alterations in the transmembrane or cytoplasmic domain of Trk A result in decreases in the degree of copatching of the receptors, when they are coexpressed in Sf9 cells. These studies also suggested that Trk A kinase activity promoted Trk A:p75 interactions, similar to the requirement for kinase activation in high affinity site formation described here (Fig. 4). However, these investigators also detect potential interactions of the extracellular domains of the receptors by the co-patching technique. In studies examining interactions between Trk B and p75, using co-immunoprecipitation analysis (14) both the intracellular domains and extracellular domains contributed to receptor complex formation. These modest contradictions with the current study may reflect, in part, the differences in techniques used to assess potential receptor interactions. The copatching and photobleaching recovery techniques are extremely sensitive in detecting biophysical interactions in living cells, yet do not yield any information as to whether these interactions could augment, or inhibit, ligand recognition and the affinity of ligand binding. Indeed, a small conformational change induced by the interaction of distant structural domains could induce a large effect on the ability of a protein to bind a substrate, in the case of
an allosteric enzyme, or a ligand, in the case of neurotrophin receptor. Thus, discrete mutations in the Trk A intracellular domains could result in slight rotational changes of the extracellular domains, particularly in the juxtamembrane extracellular sequences required for NGF binding. The abilities of structural mutations to act at a distance has been well documented in the biology of transmembrane receptors, with the cytoplasmic domains of the GPIIB/IIIA complex exhibiting “inside-out” modulation of ligand binding (50), and the transmembrane domain of the EGF receptor altering the affinity of interaction with EGF (51). It should also be noted that although the extracellular domain of p75 is not required for high affinity site formation, this finding does not exclude potential interactions between the extracellular domains of Trk receptors and p75, noted using different assessments of the multimeric receptor complexes (14,34).

The ability of p75 to distinguish between the transmembrane domains of Trk A and Trk B, with regards to high affinity NGF binding site formation, suggests that even between highly related receptors, transmembrane domain interactions may be restrictive. In this regard, it is notable that the transmembrane domains of Trk A and Trk B are more divergent (50% similarity) than are the cytoplasmic domains (87% similarity). Indeed, the cytoplasmic domain of Trk B is capable of substituting for the Trk A cytoplasmic domain in high affinity site formation (Fig. 3C and E). This ability of p75 to interact distinctively with Trk A and Trk B transmembrane domains is supported by the recent study of Vesa et al., in which p75 expression with Trk A augmented Trk A signaling, whereas coexpression of p75 and Trk B had a negative effect on Trk B signaling in response to its cognate ligand BDNF (52). Furthermore, co-expression of p75 with Trk B restricted Trk B activation to BDNF, with reductions in activation noted to NT-3 and NT-4 (13). In contrast, co-expression of p75 with Trk C resulted in a relaxation in the absolute specificity for NT-3. (52). Collectively, these studies suggest that p75, potentially via interactions with Trk transmembrane domains, can induce distinct alterations in Trk receptor conformation. In the case of Trk A, NGF binding affinity is enhanced; with Trk B, binding is more restrictive for BDNF; whereas with Trk C, the specificity of binding solely to NT-3 is relaxed. These results suggest that during pathological conditions which upregulate p75 in neurons which coexpress multiple Trk receptors, increased p75 expression could enhance NGF-dependent functions, inhibit BDNF responsivity, and broaden the ligand specificity of Trk C.
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References


Figure Legends

Figure 1. The ratio of expression of p75 and Trk A regulates the percentage of high affinity binding sites. Panel A: Transient expression of p75 and Trk A in Sf9 cells using baculoviral expression vectors allowed the two receptors to be expressed at varying ratios by altering the ratios of infectious virus. Cells were infected with virus, cultured for 24 hours, and harvested as described. Cell lysates containing equivalent concentrations of protein were separated by SDS-PAGE, and Western blot analysis was performed using antisera to the intracellular domain of p75 or the C-terminal tail of Trk A, as indicated. The positions of molecular weight markers are indicated in kD. Lanes B-E: cell lysates (10 µg) from Sf9 cells infected with baculoviral vectors encoding Trk A and p75, utilized in the corresponding equilibrium binding experiment B-E, and from Trk A-PC12 cells (50 µg) (F) (42), were probed in Western blot analysis using the indicated antiserum. Panel B-F: Equilibrium binding of 125I-NGF to cell membranes. Membrane preparations were incubated with increasing concentrations of labeled NGF (0.0005 to 4 nM), in the presence or absence of excess unlabeled ligand. Values represent means of triplicate determinations of specific binding, which averages 60-85%. Bound counts are represented as fmol. The protein content of the membranes added to each condition was 1.0 µg (20:1 ratio and 1:15 ratio) and 1.5 µg (1:5, and 1:0.8 ratios).

Figure 2. The transmembrane and cytoplasmic domains of Trk A effect high affinity site formation. To assess the roles of the transmembrane and cytoplasmic domains of Trk A in regulating high affinity site formation, a series of chimeric Trk A: torso receptor tyrosine kinase chimeric proteins were generated, and coexpressed with p75. Panel A: Schematic representations of the chimeric receptors, with Trk A sequence indicated in white, torso sequence in black, and p75 sequence by hatched lines. Panel B: Western blot analysis to assess the expression of Trk or p75 expression in membrane lysates of Sf9 (5 µg) cells infected with baculoviral vectors encoding Trk:torso chimeras and p75. Samples of Trk-PC12 cells probed in parallel confirmed receptor expression levels (data not shown). (A), wt-Trk + p75; (B), 305 + p75; (C), 303 + p75; (D), 325 + p75. The position of the molecular weight standards is indicated in kD. Panel C, D, E, F: Equilibrium binding of 125I-NGF binding to cell membranes expressing the indicated receptor constructs. Membrane preparations were incubated with increasing concentrations of labeled NGF (0.0005 to 4 nM), in the presence or absence of excess unlabeled ligand. Values represent means of triplicate determinations of specific binding, which averages 60-75%. Bound counts are represented as fmol, and membranes containing 0.1 µg of
protein were used in each assay condition.

**Figure 3. Chimeric Trk A:Trk B receptors define the IgG C2 and transmembrane domains of Trk A as sufficient to confer high affinity binding when co-expressed with p75.** Panel A: Schematic representation of the Trk A:Trk B chimeric receptors. Trk A sequence is indicated in white, Trk B sequences in black and p75 sequences in hatched lines. Panel B: Western blot analysis of 293 cells transfected with cDNAs encoding the chimeric Trks and p75. (A) Trk-PC12 cells (53); (B), 3.3 + p75; (C), 3.2 + p75; (D) Trk A + p75. Following SDS-PAGE, immobilized proteins were blotted with the indicated antisera, and the position of the molecular weight standards are indicated in kD. Panels C, D, E: Equilibrium binding of $^{125}$I-NGF using the whole cell binding assay. Cells resuspended at 0.75 x 10$^6$/ml were incubated with increasing concentrations of labeled NGF, in the presence or absence of excess unlabeled ligand. Values represent means of triplicate determinations of specific binding, which averaged 80-90%. Bound counts are represented as fmol.

**Figure 4. Trk kinase activity regulates high affinity site formation.** PC12$^{nnr5}$ cells stably expressing a kinase inactive mutant of human Trk A (K538N) were utilized to assess whether Trk A kinase activity regulates high affinity site formation. Panel A: Western blot analysis of cell lysates from K538N expressing PC$^{nnr5}$ cells (A), or Trk A overexpressing PC12 cells (clone 615)(42) (B) confirms the expression of both Trk A and p75, using the antisera described in fig 1. Panel B: Equilibrium binding of $^{125}$I-NGF binding to cell membranes. Membrane preparations were incubated with increasing concentrations of labeled NGF, in the presence or absence of excess unlabeled ligand. Values represent means of triplicate determinations of specific binding, which averages 60-75% of total binding. Bound counts are represented as fmol, and membranes containing 2 µg of protein were used in each assay condition.

**Figure 5. Domains of p75 which contribute to high affinity site formation.** Chimeric p75 receptors were generated by exchanging the extracellular and transmembrane domains of the EGF receptor for the extracellular and transmembrane domains of p75. Panel A: Schematic representation of chimeric receptors. Trk A sequence is indicated in white, EGF receptor sequences in black and p75 sequences in hatched lines. Panel B: Sf9 cells were infected with baculovirus encoding the EN10 or EN31 p75 receptors and virus encoding Trk A, and the levels of receptor expression assessed by Western blot analysis, with the positions of molecular weight standards indicated in kD. (A), Trk + EGFR-
p75(10); (B), Trk + EGFR-p75 (31). Samples of Trk-PC12 cells probed in parallel confirmed receptor expression levels (data not shown). Panel C,D: Equilibrium binding of 125I-NGF to cell membranes. Membrane preparations were incubated with increasing concentrations of labeled NGF, in the presence or absence of excess unlabeled NGF. Values represent means of triplicate determinations of specific binding, which averaged 65-80% of total binding. Bound counts are represented as fmol, and 0.5 µg protein was used per assay condition.

Figure 6. NGF binding to p75 is not required for high affinity site formation. Panel A: A mutant p75 receptor, p75 (50) incapable of binding to NGF due to a five amino acid deletion in the third cysteine repeat (36), was utilized in coexpression studies with Trk A. Panel B: Western blot analysis of 293 cells expressing Trk A and p75 (50) (A) and Trk-PC12 (clone 615)(B) was utilized to confirm the level of Trk A and p75 expression. The position of molecular weight standards, indicated in kD is shown. Panel C: Cells were incubated with radioiodinated NGF with or without unlabeled NGF, as indicated, prior to crosslinking of NGF to the cell surface of Trk A-PC12 cells (clone 615) or 293 cells coexpressing Trk A and p75 (50) using EDC. Cell lysates were separated by SDS-PAGE, and autoradiography was performed. The molecular size of approximately 90 kD is consistent with the predicted molecular wt of NGF and p75 (13 kD plus 75 kD). Panel D: Whole cell equilibrium binding studies were performed as described in figure 4 using 0.75 x 10^6 cells/ml. Specific binding averaged 75-85% of total binding, with bound counts represented as fmol. High affinity NGF binding sites are generated by cells co-expressing Trk A and the p75 (50) mutant incapable of binding NGF. Equilibrium binding studies performed using 293 cells transiently expressing Trk A alone yield low affinity binding sites.

Table 1. Schematic representation of the structural domains of the p75 and Trk A receptor required for high affinity NGF binding. Panel A: The cytoplasmic and transmembrane domains of Trk A contribute to high affinity site generation, upon co-expression with native p75. White areas, native Trk A; hatched areas, torso; black areas, Trk B. Panel B: The transmembrane domain, but not the extracellular domain of p75 is required for high affinity site generation, upon co-expression with Trk A. White areas, p75; hatched areas, EGFR; line, extracellular mutation in p75 which impairs NGF binding. The mutant p75 "lack tail" refers to results obtained with a p75 construct encoding only the membrane proximal eight amino acids of the cytoplasmic tail (38).
Table 2. Binding constants and quantitation of binding sites obtained from equilibrium binding sites. The PRISM program was used for linear regression analysis of the equilibrium binding data, and Scatchard transformation was performed to generate the $K_d$ and $B_{\text{max}}$ of each site. In experiments using membrane preparations, the $B_{\text{max}}$ is recorded in fmol/µg, denoted as (a), whereas in experiments using whole cell binding assays the $B_{\text{max}}$ is recorded in fmol/10⁶ cells, denoted as (b). Curve fits obtained by nonlinear regression analysis were compared to determine if they were similar within 95% confidence limits. The number of experiments analyzed per condition were analyzed, and conditions which were different ($p < 0.05$) from the input curve (*) are denoted (**) . (A) Fig 1; (B) Fig 2; (C) Figs 3 and 4, and (D) Figs 5 and 6.
### Table 1

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### Table 2

**Analysis of Equilibrium Binding**

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The cytoplasmic and transmembrane domains of the p75 and Trk A receptors regulate high affinity binding to NGF
Darren Esposito, Pulin Patel, Robert M. Stephens, Pilar Perez, Moses V. Chao, David R. Kaplan and Barbara L. Hempstead

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