

# Structural and Mechanistic Insights into Nerve Growth Factor Interactions with the TrkA and p75 Receptors

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## SUMMARY

Nerve growth factor engages two structurally distinct transmembrane receptors, TrkA and p75, which have been proposed to create a “high-affinity” NGF binding site through formation of a ternary TrkA/NGF/p75 complex. To define a structural basis for the high-affinity site, we have determined the three-dimensional structure of a complete extracellular domain of TrkA complexed with NGF. The complex reveals a crab-shaped homodimeric TrkA structure, but a mechanism for p75 coordination is not obvious. We investigated the heterodimerization of membrane-bound TrkA and p75, on intact mammalian cells, using a  $\beta$ -gal protein-protein interaction system. We find that NGF dimerizes TrkA and that p75 exists on the cell surface as a preformed oligomer that is not dissociated by NGF. We find no evidence for a direct TrkA/p75 interaction. We propose that TrkA and p75 likely communicate through convergence of downstream signaling pathways and/or shared adaptor molecules, rather than through direct extracellular interactions.

## INTRODUCTION

Nerve Growth Factor is the founding member of the neurotrophins, which are a family of secreted proteins essential for the proper development, patterning, and maintenance of the mammalian nervous system (Bothwell, 1995; Chao, 2003; Cohen, 1959; Dechant and Barde, 2002; Roux and Barker, 2002). NGF binds to two type-I cell-surface proteins with high affinity, the p75 receptor (Johnson et al., 1986), a member of the death-promoting Tumor Necrosis Factor family of receptors; and TrkA, a receptor tyrosine kinase (Kaplan et al., 1991; Klein et al., 1991). All neurotrophins (NT-3, NT-4/5, BDNF) bind to p75, while each NT is

specific for a different Trk receptor subtype (A-, B-, and C) (Huang and Reichardt, 2003; Kalb, 2005). The ability of NGF, and its NT family relatives, to bind two receptors of such disparate structure, which are expressed on the same cell surfaces, has spurred a long-standing debate regarding the molecular mechanism of signaling via this complex receptor system (Bothwell, 1995; Chao, 2003; Huang and Reichardt, 2003; Lad et al., 2003; Roux and Barker, 2002; Teng and Hempstead, 2004).

Both TrkA and p75 receptors have nanomolar affinities for NGF, and evidence from a variety of systems has suggested they may cooperate in transducing NGF signals (Bibel et al., 1999; Huang and Reichardt, 2003; Yano and Chao, 2000). The expression patterns of these two receptors overlap extensively (Karchewski et al., 1999; Rifkin et al., 2000), and in some instances, such as the neurons of the dorsal root ganglion, TrkA is exclusively expressed with p75 (Wright and Snider, 1995). In vitro, neurons coexpressing p75 and TrkA respond to lower concentrations of NGF than cells expressing TrkA alone (Barker and Shooter, 1994; Hantzopoulos et al., 1994; Verdi et al., 1994). p75 knockout mice require higher concentrations of NGF than normal for survival (Lee et al., 1992; von Schack et al., 2001). When the two receptors are coexpressed, the rate of association of NGF with TrkA increases (Mahadeo et al., 1994), and the presence of p75 has been reported to modulate the neurotrophin selectivity of Trk receptors. Using Scatchard analysis, it has been shown that coexpression of p75 and TrkA appears to result in the formation of a “high-affinity” binding site that has a 30- to 100-fold higher affinity for NGF than either receptor alone (Hempstead et al., 1991). On the other hand, the high-affinity binding sites appear to constitute only 10%–15% of the total NGF binding sites on cells transfected with both receptors, and several reports find no evidence for p75 influencing TrkA binding to NGF (Bothwell, 1995; Jing et al., 1992; Klein et al., 1991).

While many biochemical and cellular studies indicate the involvement of both receptors in NGF signaling, the mechanism for how these two receptors coordinate to produce a seemingly synergistic effect has been difficult

to discern. The most prevalent explanation has been that high-affinity binding is achieved through the formation of a p75/TrkA heterocomplex, perhaps involving allosteric communication between the receptors (Hempstead et al., 1991; Huang and Reichardt, 2003; Lad et al., 2003; Mischel et al., 2001; Yano and Chao, 2000). Although some groups can crosslink and/or coimmunoprecipitate a small fraction of these complexes from cells (Bibel et al., 1999; Huber and Chao, 1995; Lad et al., 2003), others have failed to observe the interaction (Jing et al., 1992). Similarly, definition of the protein domains necessary for the formation of the high-affinity binding sites on p75 or TrkA have led to conflicting results (Battleman et al., 1993; Bilderback et al., 2001; Esposito et al., 2001; Hempstead et al., 1990) (Mischel et al., 2001), thus failing to provide a molecular basis for the formation of the high-affinity sites (Bothwell, 1995; Huang and Reichardt, 2003). An alternative hypothesis is that p75 indirectly influences Trk by binding NGF at the cell surface and raising the local effective NGF concentration for TrkA (Barker and Shooter, 1994).

Current structural inferences can be derived from a homodimeric (2:2) structure of NGF complexed with a single C-terminal immunoglobulin ligand-binding domain (D5 or Ig-C2) of TrkA (Wiesmann et al., 1999). However, some debate persists as to whether this single domain is a faithful representation of the full-length receptor extracellular domain (ECD) interaction (Arevalo et al., 2000, 2001; MacDonald and Meakin, 1996; Perez et al., 1995; Windisch et al., 1995a, 1995b; Zaccaro et al., 2001). Recently, the crystal structure of NGF complexed to rat p75 revealed an unusual asymmetric stoichiometry which left one face of NGF open (He and Garcia, 2004). This observation raised the question of whether this open face could engage a TrkA ECD to form a TrkA/NGF/p75 ternary complex. Since the domains that were implicated in mediating TrkA/p75 interactions were not included in the original NGF/TrkA-D5 crystal structure, it has not been possible to evaluate a structural model of the complete receptor complexes.

In order to gain insight into the possible interaction of TrkA and p75, we have solved the crystal structure of the complete extracellular domain of TrkA complexed with NGF and evaluate the possibility, through molecular modeling, of a TrkA/NGF/p75 complex mediated by the ECDs. To further investigate the prospect of a TrkA-p75 complex on a cell surface, we employed a recently developed enzyme complementation system to detect protein interactions that can be performed on intact cells, in which the receptor transmembrane (TM) domains are included (Wehrman et al., 2005). Finally, we employed Scatchard analysis of cells transduced with similar levels of TrkA and p75, as well as the neuronal cell line PC12, to investigate the presence of high-affinity binding sites. In summary, the structural and cellular data collectively point to an alternative mechanism of TrkA/p75 communication than one involving direct receptor-receptor interaction or the formation of a ternary complex.

**Table 1. Crystallographic Statistics**

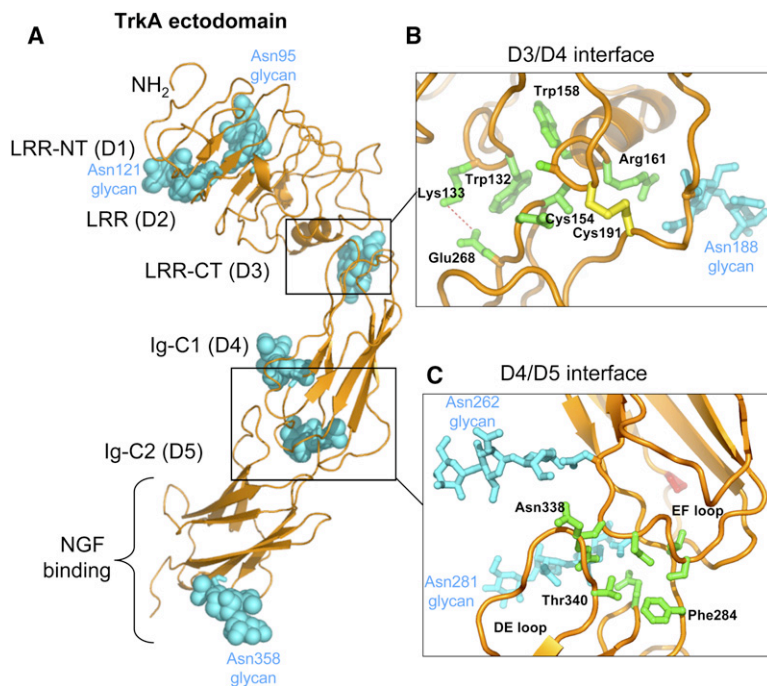
Data Collection	
Wavelength (Å <sup>2</sup> )	1.0597
Resolution (Å <sup>2</sup> )	50–3.4 (3.5–3.4)
Completeness (%)	99.0 (97.3)
I/σ (I)	9.2 (2.4)
Redundancy	4.8 (4.7)
R <sub>merge</sub> (%)	8.3 (34.9)
Model Building and Refinement	
Resolution range (Å <sup>2</sup> )	50–3.4 (3.5–3.4)
No. of reflections	26668
R <sub>cryst</sub>	0.297 (0.370)
R <sub>free</sub>	0.331 (0.391)
Average B factor (Å <sup>2</sup> )	45.2
R.m.s.d. bond length (Å)	0.008
R.m.s.d. bond angle, dihedral, improper (°)	1.6, 25.5, 0.9
Ramachandran (favored, allowed, generous, disallowed) (%)	71.9, 23.0, 5.1, 0

## RESULTS

### Structure of the TrkA Ectodomain Complex with NGF

Crystals of the complex between the TrkA ECD and human NGF were prepared using a soluble, baculovirus-expressed human TrkA ectodomain (residues 33–423) and recombinant human NGF expressed from *E. coli*. Although the crystals diffracted to moderate resolution, we avoided strategies to deglycosylate TrkA in order to improve diffraction since there have been reports that TrkA Asn-linked glycosylation can regulate its bioactivity (Watson et al., 1999). The NGF homodimer and the TrkA Ig-C2 domains in the complex were located by molecular replacement, whereas the remaining domains of TrkA were traced into electron density maps calculated using partial phases from the NGF/TrkA-Ig-C2 model (Table 1). The asymmetric unit contains a full 2:2 NGF/TrkA complex. The final TrkA structure in the refined model contains the leucine-rich repeats capped with cysteine-rich domains (D1, D2, and D3 domains, respectively) and the Ig-C1 and Ig-C2 domains (D4 and D5, respectively) (Figures 1A and 1B). Of the 13 possible N-linked glycosylation sites in each TrkA monomer, six high-mannose-containing N-linked glycosylation sites were clearly defined in the electron density in each receptor (Asn95, Asn121, Asn188, Asn262, Asn281, Asn358) (Figure 2 and Figure S1). The high-mannose carbohydrate moieties appear to play a role in the TrkA structural integrity, as several are located at TrkA interdomain boundaries. However, they do not contact the ligand and would therefore appear to influence TrkA activation only indirectly.





**Figure 2. Structural Anatomy of the Glycosylated TrkA Extracellular Domain**

(A) Complete ECD, in orange cartoon representation with Asn-linked glycosylations in cyan space-filling representation. (B) The interdomain boundary between the LRR and Ig-C1 (D4) domain, indicating the position of the disulfide bond that would constrain any hinge movements. (C) The interdomain boundary between Ig-C1 and Ig-C2 domains. Note: the interdomain insets are not in the identical orientation as panel (A) in order to achieve a clear view of the interdomain boundaries. Panel (B) has been rotated approximately 30° clockwise into the plane of the page, and panel (C) has been rotated approximately 90° counterclockwise out of the plane of the page.

related to one another by an approximately 90° twist with respect to the directions of the  $\beta$  strands (Figure 2C). The interface is primarily composed of van der Waals contacts formed by a hydrophobic cluster between the Ig-C1 EF-loop (residues 255–261) and the Ig-C2 domain BC- (residues 306–310) and DE-loops (residues 337–341). Another large Asn(281)-linked glycan from the base of the Ig-C1 domain packs into a groove on the side of the Ig-C2 domain, supplementing the already intimate interdomain boundary. Thus, both the LRR/Ig-C1 and Ig-C1/Ig-C2 boundaries appear highly structurally constrained, and it appears unlikely that large-scale conformational changes would occur upon binding to NGF, as has been hypothesized in an allosteric model of TrkA activation (Zaccaro et al., 2001).

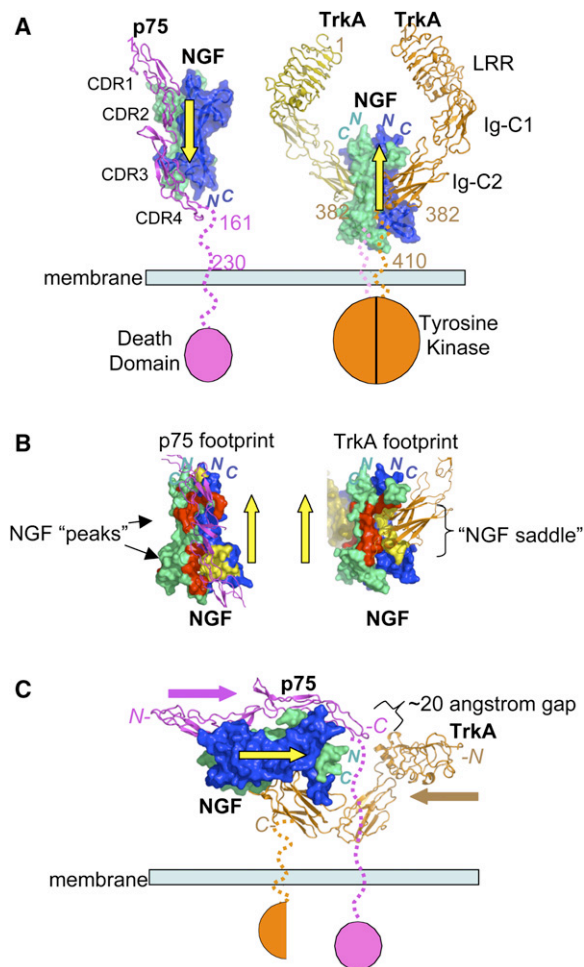
Of the five TrkA domains, only the C-terminal Ig-C2 domain interacts with NGF in the complex structure, as originally seen in the truncated complex structure (Wiesmann et al., 1999). The interaction between Ig-C2 and NGF is rather striking in that the top loops of the Ig-domain penetrate a saddle-like depression along the tops of the NGF central  $\beta$  sheets, in an almost orthogonal fashion (Figure 3B). As the NGF/TrkA-D5 interaction shows no substantial deviations from the previous structure, we will not describe the interface exhaustively.

One impetus for determining the structure of a full-length TrkA ECD complex with NGF was that controversy still persists over whether the Ig-C2/NGF complex represents the bona fide receptor complex, as several studies have implicated additional N-terminal domains of TrkA in NGF recognition (see Discussion) (Arevalo et al., 2000, 2001; MacDonald and Meakin, 1996; Perez et al., 1995; Windisch et al., 1995a, 1995b; Zaccaro et al., 2001). The

full-length TrkA ECD structure clearly shows that only the Ig-C2 domain contacts NGF, and this observation almost certainly extends to the other TrkA receptors (Figure 1) (Urfer et al., 1998). We have carried out companion solution studies and find that the stoichiometry of this complex is 2:2 even at saturating NGF concentrations, further suggesting a single NGF binding site on TrkA (data not shown). The structural and biochemical data strongly argue for the fact that NGF does not contact any domains in the TrkA ECD other than Ig-C2.

The second issue we hoped to gain some structural insight into was whether there could be an interaction between the ECD of TrkA and p75 in a putative ternary complex with NGF. Numerous studies have suggested that a direct interaction between TrkA and p75 is necessary for the modulation of affinity and specificity observed when both receptors are coexpressed (see Discussion) (Hempstead et al., 1991; Lad et al., 2003; Mischel et al., 2001; Zaccaro et al., 2001). Using information gained from the structure of the full-length TrkA ECD, our intention was to assess the likelihood of a ternary complex of Trk/NGF and p75.

We recently determined the structure of the rat p75 extracellular domain complexed with human NGF (He and Garcia, 2004) (Figure 3A). This structure showed that the NGF/p75 docking mode is completely different than NGF/TrkA (Figures 3A and 3B). The structure also displayed an asymmetric binding mode in which only one side of the NGF dimer was bound to p75 (Figure 3A). The asymmetric stoichiometry was apparently induced by NGF binding and was verified as a stable entity in solution (He and Garcia, 2004). A recent study has suggested that this 1:2 complex could be an intermediate on the path toward



**Figure 3. Comparative Structural Analysis of NGF/p75 and NGF/TrkA Complexes**

(A) The structure of NGF complexed to p75 (He and Garcia, 2004) and the neighboring complex of NGF with TrkA. The polarity of NGF in the two complexes is indicated with yellow arrows (pointing toward N and C termini). The intracellular domains of both receptors are drawn schematically. NGF is depicted with molecular surfaces, while p75 and TrkA are shown as ribbons. Note the open face of NGF on the asymmetric p75 complex that could represent a potential binding site for a single TrkA ectodomain in a scenario where p75 and TrkA existed as an equilibrium of intermediate homo- and heterocomplexes on the cell surface. (B) The binding sites of NGF on p75 and TrkA are mutually exclusive on the same face of NGF, but the binding footprints are largely nonoverlapping on the NGF surface. The red and yellow patches on NGF indicate the portions of the binding surface on each NGF monomer; note how both Trk and p75 contact both halves of the homodimer. (C) A ternary complex of a p75/NGF/TrkA 1:2:1 heterotrimer is sterically feasible if the receptors lie parallel to the membrane in order to sandwich NGF between them in antiparallel orientations. The closest point of approach between p75 and TrkA is between the base of the p75 ECD and the LRR of TrkA, where they are within 20 Å reach.

formation of a stoichiometric 2:2 complex, but this has not been unequivocally demonstrated (Aurikko et al., 2005). One explanation for the 1:2 stoichiometry and asymmetry of the NGF/p75 complex crystal structure was to maintain

an open face of NGF for formation of a ternary complex with TrkA (He and Garcia, 2004). Importantly, the conformational changes induced in NGF by p75 did not extend to its TrkA binding surface. Now, with structures of both NGF/p75 and NGF with full-length TrkA ectodomain, we can model a ternary complex.

When modeled on the cell membrane and compared side by side, two features are apparent. First, NGF binds to TrkA and p75 in opposite orientations (Figure 3A). When bound to TrkA, the “head” of NGF (N and C termini) is membrane proximal, but when bound to p75, the head is membrane distal. Therefore, TrkA and p75 will be in an antiparallel orientation if both are simultaneously bound to NGF. Second, TrkA and p75 interact with NGF through nonoverlapping binding sites that are on the same face of NGF. p75 forms a two-point attachment along protruding surfaces of the NGF homodimer by docking within a groove between monomers (Figure 3B). In contrast, TrkA-D5 binds within the saddle of NGF, between the protruding surfaces used by p75 (Figure 3B). Even though the “footprint” of the binding sites are largely nonoverlapping, p75 and TrkA would sterically obstruct one another from binding simultaneously to the same face of NGF. Therefore, in accord with the modeling studies of Aurikko et al., there does not appear to be a possibility of a 2:2:2 symmetric tricomplex of TrkA/p75/NGF (Wiesmann and de Vos, 2001). However, p75 and TrkA can bind to opposite sides of NGF simultaneously to form a 1:2:1 ternary complex without any steric clashes (Figure 3C).

Given the two constraints mentioned above (antiparallel orientation and mutually exclusive binding sites on NGF), it is sterically possible to construct a ternary complex (Figure 3C). However, this cannot be accommodated if the receptors both extend straight up from the cell surface but can be easily accommodated if they lie parallel to the membrane, sandwiching NGF between them. There is ample precedent for receptors engaging ligand parallel to the membrane (Chow et al., 2001), and this is spatially possible for TrkA and p75 since they both have extended connecting peptides linking the ECD to the cell surface that likely permit flexibility. In this ternary complex, the TrkA N-terminal LRR is nearest to the C-terminal base (CRD1) of p75, but the domains are separated by approximately 20 Å (Figure 3C). Direct contact between the receptors would require a rotation of the LRR around the linker connecting it to the D4 domain. As the interdomain hinges appear constrained by a disulfide bond (Figure 2B), we are skeptical whether such a conformational change would occur to result in p75/TrkA contact.

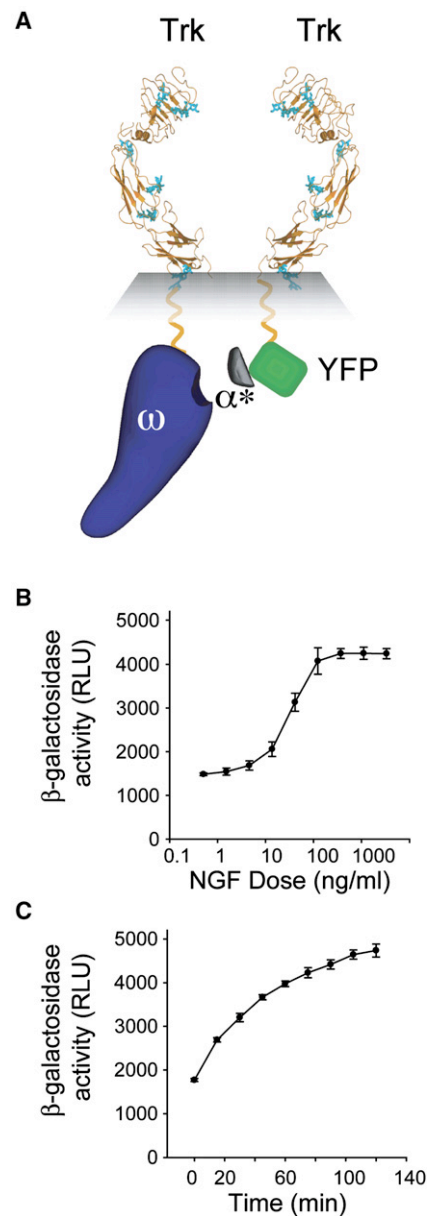
We have been unable to produce a stable TrkA/p75/NGF ternary complex in solution. One possible explanation for this is that the transmembrane regions of the receptors play a role in p75/TrkA interactions, and they are missing in the soluble ECD constructs, as suggested in one study. Therefore, we decided to assess TrkA/p75 interactions on living cells in a format in which the TM segments are preserved, as well as portions of the intracellular domains.

### TrkA/p75 Interactions Assayed in a Cellular Context

To determine potential interactions of TrkA and p75 in intact cells, we utilized a previously described complementation system of  $\beta$ -galactosidase to address this question (Wehrman et al., 2005, 2006). The general principle is that  $\beta$ -galactosidase is split into two inactive fragments, a small fragment ( $\alpha$ ) and a larger fragment ( $\omega$ ), which, when fused to two proteins that interact, reconstitute into an intact enzyme with activity that can be easily measured by luminescence (e.g., Figures 4A and 5A). This  $\beta$ -gal method is a weakly complementing protein interaction system (i.e., low background activity) wherein the enzyme activity is directly proportional to the local concentration of the two enzyme fragments. The system was first used to monitor changes in local protein concentration associated with intracellular protein movement (protein translocation) (Wehrman et al., 2005, 2006). As the concentration of  $\alpha^*$  or  $\omega$  increases, the enzyme activity increases accordingly. A significant advantage of enzyme complementation over studies using purified proteins or coimmunoprecipitation is that the receptors remain in the two-dimensional context of the plasma membrane as opposed to the three-dimensional environment obtained in solution or in cell lysates. Most importantly, a *specific* binary interaction of  $\alpha^*$  and  $\omega$  is necessary to obtain  $\beta$ -galactosidase activity, thus providing a direct “molecular” or “stoichiometric” assessment of the protein-protein interaction. This feature of the technique differs substantially from co-IP and crosslinking, which assess the proximity of neighboring molecules that could be associated for reasons other than a specific interaction.

The ECD and TM regions of TrkA (TrkA) and p75 as well as the full-length p75 (p75FL) were each fused to the N terminus of  $\omega$  and YFP-H31R  $\alpha$ -peptide fusion ( $\alpha^*$ ). Since TrkA is efficiently internalized upon activation, if the full-length TrkA receptor were used in the  $\beta$ -gal system, then the internalization would result in an artifactual decrease in the concentration of the receptors at the plasma membrane and thus result in a change in  $\beta$ -gal activity independent of ligand interaction. In order to avoid modulation of  $\beta$ -gal activity that was not associated with receptor interaction, we utilized a truncated TrkA receptor that eliminates the internalization motifs. In contrast, p75 is not actively internalized, so both full-length and truncated versions of the receptor could be studied. Therefore, all of the constructs in these experiments contain at least both ECD and TM regions, which have been implicated in mediating the TrkA/p75 interaction (Esposito et al., 2001; Mischel et al., 2001). Prior to these experiments we ascertained that C2C12 cells do not express significant amounts of p75 by Western blotting untransfected C2C12 cells and comparing to the transfected C2C12 cells (Figure S3).

TrkA is well known to homodimerize in the presence of NGF; thus we determined whether this interaction could be measured using  $\beta$ -gal complementation. A stable cell line containing the TrkA $\omega$  and TrkA $\alpha^*$  fusion constructs was generated by retroviral infection of C2C12 cells (Figure 4A). Cells were plated into a 96-well dish and as-



**Figure 4. Homodimerization of TrkA Monitored in Mammalian Cells using  $\beta$ -Gal Complementation**

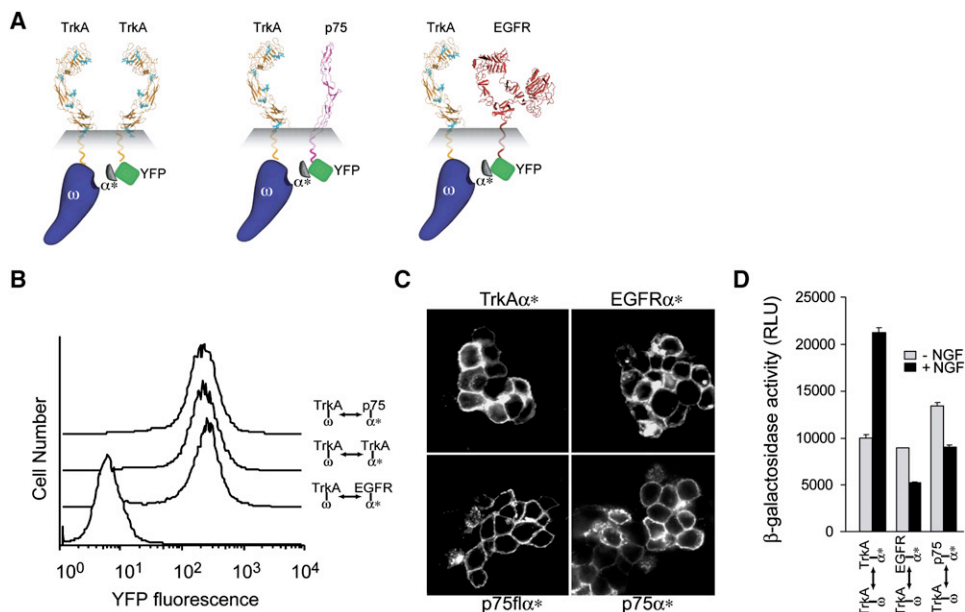
(A) The low-affinity  $\beta$ -gal complementation system consists of a mutated  $\alpha$  peptide ( $\alpha^*$ ) and the traditional  $\omega$  fragment that are fused to the two proteins that interact. Interaction of the two proteins results in an increase in enzyme activity.

(B) C2C12 cells expressing TrkA- $\alpha^*$  and TrkA- $\omega$  were exposed to increasing doses of recombinant NGF, and  $\beta$ -gal activity was measured using a chemiluminescent substrate.

(C) A time course of TrkA homodimerization was obtained by exposing the cells to 1  $\mu$ g/ml NGF at different times.

Error bars represent one standard deviation.

sayed in triplicate for changes in  $\beta$ -gal activity in the presence of NGF. Addition of NGF to the medium increased  $\beta$ -gal activity by greater than 250%, indicating robust



**Figure 5. The Interaction of TrkA with p75 Is Not Detectable using  $\beta$ -Gal Complementation in the Presence or Absence of Ligand** (A) The cell lines constructed with the indicated receptor pairs are shown as fusions to the  $\alpha^*$  and  $\omega$  fragments.

(B) To control for protein expression levels, a parental cell line was constructed that stably expressed TrkA- $\omega$ . This line was transduced with the TrkA- $\alpha^*$ , p75 $\alpha^*$ , and EGFR $\alpha^*$ . Because each  $\alpha^*$  construct contains YFP inserted between the receptor and the  $\alpha^*$ , cells were sorted by FACS for similar expression levels.

(C) The localization of each of the  $\alpha^*$  constructs must be similar in order for the flow cytometry data to be an accurate representation of the protein expressed at the cell surface. Transfection of the  $\alpha^*$  constructs into HEK293 cells shows a similar membrane staining pattern for all of the receptor fusions.

(D) The cells expressing each of the receptor pairs were plated at the same density in a 96-well dish and assayed for  $\beta$ -gal activity in the presence and absence of 1  $\mu$ g/ml NGF. Each cell line was assayed in triplicate with error bars representing one standard deviation. The TrkA is shown to homodimerize efficiently, whereas both the control cell line, TrkA-EGFR, which should not interact, and the TrkA-p75 line both show a decrease in  $\beta$ -gal activity in the presence of NGF, presumably due to homodimerization of the TrkA that reduces the basal enzymatic activity obtained. The basal enzyme activity for each cell line is similar, indicating similar levels of spontaneous interaction for all receptor pairs tested.

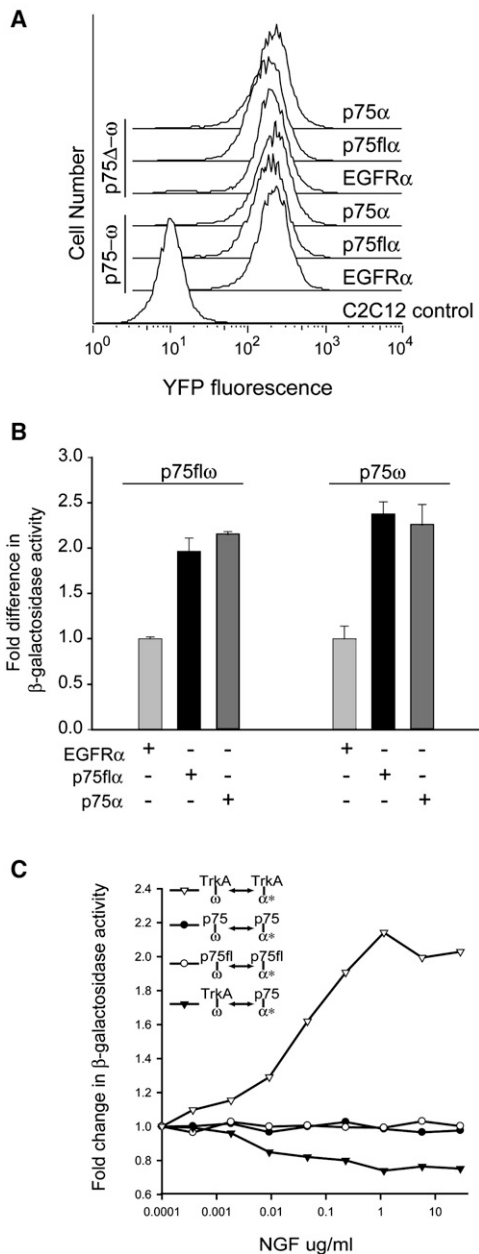
homodimerization of TrkA. This effect is dose and time responsive, demonstrating specificity of the reaction (Figures 4B and 4C).

Mechanistically, TrkA and p75 could cooperate in many different ways. The proteins might assemble spontaneously and dissociate in the presence of ligand, or heteromeric complexes could form as a result of ligand binding. Because the stoichiometry and inducibility of a p75-TrkA complex is unknown, it was necessary to study NGF-induced changes in protein interaction as well as basal levels of dimerization. In order to design a system that could measure background as well as induced interaction, we normalized the expression level of the fusion proteins because the overall enzyme activity derived using the  $\beta$ -gal system is sensitive to the expression levels of the two enzyme fragments. To control for fusion protein expression levels, a parental cell line was constructed stably expressing TrkA $\omega$ . The cell line was then transduced with EGFR $\alpha^*$ , TrkA $\alpha^*$ , and p75 $\alpha^*$  (Figure 5A). YFP was included between the receptor domain and the  $\alpha^*$  to enable selection of cells expressing similar levels of  $\alpha^*$  by FACS and to present the  $\alpha$  peptide similarly in all fusion proteins. Because each cell line is derived from the same parental

population, the levels of  $\omega$  are equivalent. FACS was used to sort for YFP expression, which correlates with expression of the  $\alpha^*$  (Figure 5B). In this manner the expression of each of the  $\beta$ -gal fusions has been normalized. In order for the total YFP fluorescence to accurately reflect the concentration of the  $\alpha^*$  fusions, the proteins must be similarly localized. Transfection of each construct into HEK293 cells shows a predominant membrane localization for all constructs tested (Figure 5C).

The resulting three cell lines were plated at the same density in a 96-well dish and assayed for  $\beta$ -galactosidase activity in the absence and presence of NGF. The background activity of the system is defined by the amount of signal generated from the noninteracting protein pair, TrkA $\omega$ -EGFR $\alpha$ , which results from a weak spontaneous complementation of the fragments. As expected, the TrkA $\omega$ -TrkA $\alpha^*$  cell line showed an increase in enzyme activity induced by the presence of NGF. The background activity of this cell line and the TrkA $\omega$ -p75 $\alpha^*$  is similar to the control TrkA $\omega$ -EGFR $\alpha^*$  cell line, indicating little, if any, basal dimerization of these receptors (Figure 5D).

The EGFR-TrkA cell line shows no induction in the presence of NGF, nor does TrkA and p75, demonstrating that



**Figure 6. p75 Oligomerizes at the Cell Surface and the Association Is Not Affected by NGF Binding**

(A) Parental cell lines were created using the full-length (p75FL) and truncated (p75) forms of the p75 receptor fused to the  $\omega$ . The parental cell lines were transduced with the indicated  $\alpha^*$  fusion proteins and sorted for similar YFP expression levels as a measure of the  $\alpha^*$  present in each line.

(B) Cells expressing the indicated  $\beta$ -gal receptor fusions were assessed for their basal enzymatic activity. Each cell line is shown as a fold increase over the control cell line which should not interact (EGFR-p75), with error bars representing one standard deviation. The lines expressing either a truncated or full-length p75 show a 2-fold higher  $\beta$ -gal activity than the control EGFR-p75 cell lines, indicating that p75 homo-oligomerizes. Both the truncated and full-length receptors show similar levels of enzyme activity, demonstrating that the extracellular and transmembrane domains are sufficient for the interaction.

p75 and TrkA do not inducibly form heterodimers. In both the TrkA $\omega$ -EGFR $\alpha^*$  and TrkA $\omega$ -p75 $\alpha^*$  cell lines, a modest decrease in enzyme activity is observed. The loss of enzyme activity suggests that NGF is homodimerizing the TrkA fusion and thus removing TrkA from the pool, therefore limiting the basal interaction of the TrkA $\omega$ -p75 $\alpha^*$  enzyme fragments. Because no induction of enzyme activity was observed for the p75 construct, we confirmed that cells expressing p75 were able to bind NGF using a fluorescently labeled NGF (Alexa 647) and analysis by flow cytometry (Figure S2). As previously mentioned, we also verified that C2C12 cells do not express levels of endogenous p75 that would effect the  $\beta$ -gal readout (Figure S3). These data provide evidence for an NGF-inducible interaction of TrkA homodimers, but not TrkA and p75 heterodimers.

### Homotypic p75 Interactions

Several reports have shown that NGF binding to p75 initiates an apoptotic cascade in the absence of TrkA (Frade et al., 1996; Majdan et al., 1997), although the molecular basis of this observation remains unclear. Although TNF-family death receptors normally exist as trimers, quiescent p75 has been shown by crosslinking to exist as a dimer on the cell membrane (Grob et al., 1983). Binding of NGF to p75 has been variously proposed to induce the dimerization of p75 (Wang et al., 2001) or disrupt preformed p75 dimers (Wang et al., 2000) or form a range of receptor-ligand stoichiometries (Aurikko et al., 2005; Ibanez et al., 1992). To address changes in oligomerization of p75 by NGF, we first determined the basal oligomerization state of p75 by constructing six cell lines expressing pairwise combinations of the p75 (truncated), p75fl (full-length), and EGFR fused to the  $\alpha^*$  and  $\omega$   $\beta$ -galactosidase enzyme fragments. Parental cell lines were constructed using the p75fl $\omega$  and p75 $\omega$ . The p75 $\alpha^*$ , p75fl $\alpha$ , and EGFR $\alpha$  were then introduced into these cell lines (Figure 6A). Cells were sorted for similar YFP expression levels and plated at the same density in a 96-well dish. Enzyme activity was measured in the absence of NGF. For each parental cell line, the enzyme activity resulting from the EGFR construct was placed at a value of 1, and the activity of the other cell lines was expressed as a fold change (Figure 6B). All cell lines expressing either p75 or p75fl fused to the  $\alpha^*$  and  $\omega$  generate greater than 200% of the activity of the cell lines containing the EGFR. These results suggest that p75 at least oligomerizes in the absence of ligand, in accord with previous studies using purified proteins or immunoprecipitation (He and Garcia, 2004; Jing et al., 1992). Therefore, membrane-bound p75 full-length

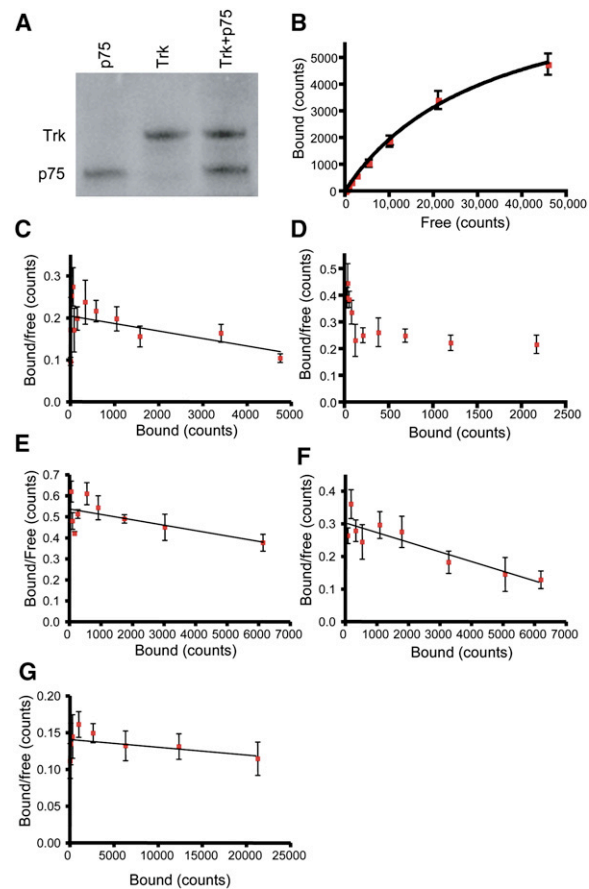
(C) The truncated p75 and full-length p75 homodimerization cell lines were tested for a change in association in the presence of the ligand NGF. As controls, the TrkA homodimerization cell line was included and shows a robust homodimerization. Similar to what was observed previously, the TrkA-p75 cell line showed a modest decrease in  $\beta$ -gal activity in the presence of NGF. Addition of NGF had no effect on the enzyme activity obtained from the p75 homodimerization cell lines.

and truncated receptors behave similarly to the soluble ECD (He and Garcia, 2004).

In order to test the ability of NGF to disrupt preformed oligomers, the p75 homodimerization cell lines were compared to the TrkA homodimerization and TrkA-p75 heterodimerization cell lines (Figure 6C). Cells were treated with various concentrations of NGF for 45 min and then assayed for changes in  $\beta$ -gal activity. In the presence of NGF, the TrkA $\omega$ -TrkA $\alpha^*$  cell line shows a dose-dependent increase in enzyme activity as expected, and the TrkA $\omega$ -p75 $\alpha^*$  cell line shows a decrease in activity as shown earlier. The p75 homodimerization cell lines show no significant change at any dose tested, indicating that NGF does not change the oligomerization state of the receptor, despite binding to the ECD (Figure S1).

### Scatchard Analysis of Cells Expressing p75 and TrkA

The results from the biochemical studies failed to support the physical association of soluble TrkA and p75 ECDs. The enzyme complementation data confirmed the existence of p75 oligomers and NGF-induced TrkA homodimerization, but a basal or induced interaction of p75 with TrkA was not observed. To further explore this possibility, we performed radiolabeled binding studies of NGF to cells transduced with TrkA $\alpha^*$  and p75 $\alpha^*$ . Western blot using an antibody against GFP shows that the cell line expresses similar amounts of each protein (Figure 7A). Cells were treated with increasing doses of 125I NGF, and the amount of bound and free ligand was determined (see Experimental Procedures). The reported number of high-affinity binding sites for NGF ranges from 5%–15% of the total NGF binding. Thus, to achieve maximum sensitivity, subsaturating doses of NGF were used (0.00001 to 1 nM/100,000 cells). The saturation binding curve indicates a  $K_d$  of  $0.9 \text{ nM} \pm 0.3$  in good agreement with the published values of the “low-affinity” NGF binding site, which is equivalent to binding to p75 or TrkA alone. The data were analyzed using nonlinear regression of one-site and two-site binding models (Figure 7B). The data could only be fit by a one-site binding isotherm yielding an  $r^2$  value of 0.9733. Background-subtracted Scatchard transformation of the binding data also shows a single linear relationship (Figure 7C). By comparison, the same data without a background subtraction shows a curvilinear plot (Figure 7D) with a trend upward at low ligand concentration, similar to that observed for the classical high-affinity site. Therefore, we can reproduce a two-site model by performing the Scatchard analysis without a background correction. We also performed background-subtracted Scatchard analysis of C2C12 cells transfected with full-length p75 and truncated TrkA alone and find the same single-site result as for the cotransfected TrkA/p75 cells (Figures 7E and 7F). In order to test whether the modified Trk and p75 constructs in the transfected cells used for Scatchard analysis were responsible for the absence of the high-affinity site, we also carried out Scatchard analysis of a neuronal cell line, PC12, which express endogenous full-length p75 and TrkA (Figure 7G). Again, we fail



**Figure 7. Scatchard Analysis of TrkA and p75 on Transfected and Neuronal Cells**

(A) Western blot analysis of C2C12 cells expressing the indicated  $\alpha^*$  fusion proteins. The TrkA $\alpha^*$ -p75 $\alpha^*$  cell line was used in the binding studies.

(B) Saturation binding curve of radiolabeled NGF binding to the TrkA $\alpha^*$ -p75 $\alpha^*$  cell line.

(C) Scatchard transformation, with background subtraction, of the binding data shown in (B).

(D) Scatchard transformation of the binding data without subtracting the background signal.

(E) Scatchard analysis of truncated TrkA $\alpha^*$  alone showing single-site binding.

(F) Scatchard analysis of full-length p75 $\alpha^*$  alone showing single-site binding.

(G) Scatchard analysis of PC12 cells showing single-site binding. Error bars represent one standard deviation.

to detect a high-affinity binding site by Scatchard transformation of the equilibrium binding data and instead determine a single affinity for NGF of approximately  $K_d \sim 1 \text{ nM}$ .

One of the problems we and others (Klotz and Hunston, 1984; Nekhai et al., 1997; Nicosia, 1988; Zierler, 1989) have observed in performing Scatchard transformation of binding data is that at low values the Scatchard manipulation is highly sensitive to errors in background calculation. As the values decrease, the scatter of the data points is not random but has a trend upward approaching a

value of 1 if the background is not correctly measured (Figure 7D). For this and other reasons, Scatchard transformation of data for radiolabeled ligand binding has been discouraged and largely replaced with nonlinear transformation of saturation binding data.

## DISCUSSION

Our goal in this report was to (1) clarify the structural biology of NGF/TrkA interactions and (2) probe a molecular basis for the apparent communication between p75 and Trk receptors. Since the discovery that NGF could bind two separate transmembrane receptor systems present on the surface of a single neuronal cell, elucidating the interplay of these molecules in neuronal signaling has been enigmatic. The literature bearing on this topic is vast, but so far inconclusive. Although most studies are in agreement that both TrkA and p75 play a role in NGF signaling, the mechanism by which p75 influences TrkA signaling remains unclear. Many published studies, citing several lines of functional, cellular, and structural evidence, propose that the receptors themselves may physically interact or communicate through allostery (summarized in Introduction) (Bothwell, 1995; Lad et al., 2003; Mischel et al., 2001; Yano and Chao, 2000). On the other hand, these data are contradicted by several studies showing that p75 has no influence on Trk binding affinity (Aurikko et al., 2005; Bothwell, 1995; Jing et al., 1992).

Since the ligand binding of both Trk and p75 are dictated by their extracellular domains, and the various Trk(A, B, or C)/p75 high-affinity binding sites are neurotrophin-specific, involvement of these domains would seem a necessary prerequisite to explain the effects of p75 on Trk ligand binding, as suggested (Lad et al., 2003). We first addressed whether additional extracellular domains of TrkA, not included in an earlier NGF complex with a single Ig-C2 domain of TrkA, could influence NGF recognition or interaction with p75. It has been proposed that neurotrophins interacted with the LRR domain of TrkA (MacDonald and Meakin, 1996; Windisch et al., 1995a, 1995b), and Zaccaro implicates both the LRR and Ig-C1 domains, in addition to Ig-C2, as being necessary for NT activation of TrkA and coordination with p75 (Zaccaro et al., 2001). Zaccaro further proposes a model whereby TrkA contains “high” and “low” affinity NGF binding sites on the ECD that are regulated allosterically through TrkA conformational changes, such as interdomain rotations. From the structure presented in this paper, it is clear that NGF only interacts with the TrkA-D5 domain, consistent with several structure-function studies using recombinant NGF and TrkA Ig-C2 domain (Urfer et al., 1998). With regards to potential NGF-induced allostery in TrkA, the domains of TrkA are associated with each other through large and constrained interdomain interfaces—including a disulfide bond between the LRR and Ig-C1 domains. It would be energetically costly to disrupt these boundaries in order to undergo significant conformational changes, although in the absence of an

unliganded TrkA structure we cannot completely rule out this possibility.

With the structure of the complete NGF/TrkA ECD complex in hand, we can now address the issue of TrkA communication with p75 with adequate structural models. Recently, the structure of p75 bound to NGF was reported, in which only a single p75 molecule was bound to one side of the NGF homodimer, leaving one face of NGF open. Conceptually, this complex structure was consistent with the observation that p75 coordinates with numerous different cell surface receptors to transduce signals in a variety of neuronal settings, such as Nogo receptor, Sortilin, and Trk (Barker, 2004). p75 and Trk could exist in a dynamic equilibrium of transient homo- and heterodimeric complexes, depending on the relative concentrations of each, that is controlled through conformational changes of p75 and Trk, as others have also suggested (Aurikko et al., 2005; Ibanez et al., 1992). From the structure of the TrkA full-length extracellular domain we see that TrkA and p75 have different binding footprints on NGF, but their binding sites overlap in a way that prevents both from binding simultaneously on the same side of NGF to form a 2:2:2 complex, as was modeled previously (Wiesmann and de Vos, 2001). Although we cannot detect association between soluble p75 and soluble TrkA ECD, the plasma membrane may be necessary for this interaction (Esposito et al., 2001).

To maintain the native state of the receptors in the plasma membrane and test the contribution of the transmembrane regions to the interaction, we used a recently developed enzyme complementation system designed to detect reversible protein-protein interactions in intact cells (Wehrman et al., 2005, 2006). Using this technique we show that the ECD and TM regions of TrkA are sufficient to mediate homodimerization in the presence of NGF as expected. Both truncated and full-length p75 were found to exist in a multimeric form both in the presence or absence of NGF, similar to findings in cells (Grob et al., 1983; Jing et al., 1992). This latter result suggests that the mechanistic role of NGF in p75 activation may be to perturb or alter the preformed dimer orientation in order to initiate intracellular signaling, rather than disrupt it. However, we were unable to find evidence for the formation of a p75 and TrkA heterodimer.

The results from all three approaches point to the fact that the ECD and TM domains of these receptors are insufficient to mediate their interaction. Because these results were surprising given the biological evidence for the interaction, we revisited the traditional radiolabeled NGF experiments to determine if the ECD and TM portions of these molecules were sufficient to form the high-affinity binding site. Binding of NGF to cells expressing both TrkA and p75 in our experiments showed a saturation binding curve that only fit a single-site model using nonlinear regression with the expected equilibrium binding constant of the “low-affinity” site. Scatchard transformation of the data for which we carried out a background correction also failed to reveal the presence of the reported

high-affinity binding site. These results confirm the hypothesis that the extracellular and transmembrane domains of Trk and p75 give rise to only a single class of low-affinity binding sites. We also carried out Scatchard analysis with background correction for C2C12 cells individually transfected with full-length p75 and truncated TrkA (Figures 7E and 7F). Again, we do not detect a high-affinity binding site, rather we measure an NGF affinity of approximately 1 nM, which is similar to that of p75 and TrkA alone for NGF. Finally, in order to account for the possibility that the modified Trk and p75 constructs we used for transfection were in some way artifactually prevented from forming a high-affinity site, we carried out NGF binding Scatchard analysis on PC12 cells expressing endogenous (and by definition full-length) p75 and TrkA, and we still do not detect the high-affinity site, but rather a single site with an affinity of  $\sim 1$  nM (Figure 7G).

Coimmunoprecipitation results have also suggested an interaction of TrkA with p75. One study showed that the interaction of p75 and TrkA was only detectable if Trk was immunoprecipitated using antibodies to the intracellular domain and not using antibodies to the extracellular domain. (Lad et al., 2003). Since TrkA is efficiently internalized in the presence of NGF, one plausible explanation is that the observed interaction comes from the immunoprecipitation of intact endosomes that are contaminated with p75, as it is not actively excluded from the endosomal compartment. Antibodies that recognize the extracellular domain of TrkA would fail to immunoprecipitate this fraction and thus failed to coimmunoprecipitate p75. Importantly, the assays employed in our work using enzyme complementation utilize a specific bimolecular interaction of  $\alpha^*$  and  $\omega$ . In order to achieve changes in enzyme activity, the  $\alpha^*$  and  $\omega$  fragments must interact directly, providing a more stringent analysis of protein interactions than coimmunoprecipitation experiments where fragments of plasma membrane or endocytic vesicles which contain both receptors will result in positive signals.

Perhaps the most compelling evidence for the collaboration of Trk and p75 stems from the biological observation that expression of both Trk and p75 enhances and sensitizes neurons to neurotrophin signaling. The presence of the high-affinity binding site for Trk and p75 provides a convenient explanation for the ability of neurons to respond to low concentrations of neurotrophin. However, the high-affinity binding site is consistently reported to be 100 $\times$  higher than the low-affinity binding observed for either TrkA or p75 alone, whereas the difference in cellular response to NGF is only on the order of 1.5- to 3-fold. The discrepancy in the affinity versus responsiveness implies that saturation of the high-affinity binding site in tissue culture cells is not sufficient to mediate a significant cellular response *in vitro*. *In vivo*, NGF is also present at extremely low concentrations and, yet, initiates signaling which again suggests a role for the high-affinity binding site. An explanation for this phenomenon can be derived from elegant work showing that Trk homodimers are capable of being internalized and continued signaling in

the endosome (Howe and Mobley, 2005; Riccio et al., 1997). The concentration of NGF in the endosome is sufficient to saturate the “low-affinity” binding sites, thus creating a prolonged signaling effect. The extraordinarily slow off rate of the TrkA/NGF complex creates a system that can maintain a prolonged response to very low levels of ligand using only the described low-affinity binding sites. The number of active receptors necessary to transmit a biological response remains to be determined, but the unusual sensitivity of the system might be explained by the stability of the TrkA signaling complex in the endosomes rather than saturation of the high-affinity binding sites.

Taken together, our structural, biochemical, and molecular data argue against a direct structural “collaboration” between p75 and TrkA in forming the high-affinity NGF binding site. However, they do not exclude the possibility that a high-affinity NGF binding complex exists. The structural data presented here discourage the possibility of an allosteric modulation of the extracellular domain of TrkA, but other mechanisms such as clustering of receptors in lipid rafts or the participation of other associated proteins may account for the observed alterations in NGF affinity. In principle, if TrkA and p75 were tethered to the cytoskeleton or the same intracellular adaptor molecule (Chang et al., 2004), it could result in an avidity effect that might appear as higher-affinity binding.

Alternatively, a model of convergent signaling between these two unrelated receptors could explain the downstream biological observations. The signaling molecules activated by TrkA are typical of conventional trophic signals transduced by receptor tyrosine kinases, such as activation of the MAPK and PI3-kinase pathways. By contrast, the list of proteins and pathways activated by p75 is much more extensive. At least nine proteins are thought to interact with the intracellular domain of the receptor that mediate apoptotic (Khursigara et al., 1999; Ye et al., 1999) as well as prosurvival signals (Lachyankar et al., 2003; Nykjaer et al., 2005; Roux et al., 2001). In addition, p75 is known to activate nf-kappaB (Burke and Bothwell, 2003; Carter et al., 1996; Hamanoue et al., 1999) and jnk (Harrington et al., 2002; Majdan et al., 1997). The variety of signaling molecules influenced by p75 makes it likely that crosstalk between these and TrkA effectors mediate the pleiotropic biological effects of p75 (Yeiser et al., 2004). In support of this hypothesis, it has been shown that separate populations of cells expressing both p75 and Trk respond differently to NGF, suggesting that the specific profile of intracellular mediators (Bilderback et al., 2001; Casademunt et al., 1999; Yeiser et al., 2004) may dictate the phenotypic response and sensitivity to NGF rather than physical collaboration of p75 and TrkA in NGF binding.

## EXPERIMENTAL PROCEDURES

### Cloning and Expression

Recombinant human nerve growth factor, expressed and refolded from *E. coli*, is a gift from Genentech, Inc. (A.M de Vos). The human TrkA receptor was produced using the baculovirus system in insect

cells (Baculogold-Pharming). We amplified and subcloned the coding sequence for the full extracellular domain (residues 33–423) into the pAcGp67A secretion vector (Pharming); 7-histidine tags were appended to the COOH-termini. Recombinant baculovirus was produced and amplified using sf9 cells in serum-containing media. For large-scale expression of the protein, 1 l of Hi5 cells were grown to a density of  $1.8 \times 10^6$  cells per milliliter in Insect-Xpress (BioWhittaker) in a shaking Fernbach flask and were infected with recombinant virus at an MOI of  $>10$ . Cultures were allowed to progress for 66 hr before the cells were pelleted by centrifugation; the supernatant was concentrated and exchanged into a buffer of HBS (HEPES-buffered saline, 10 mM HEPES [pH 7.2], 150 mM NaCl), captured by Ni-NTA resin, and then purified with gel-filtration chromatography (Superdex-200). The NGF/TrkA complex was prepared by mixing excess amount of NGF with TrkA and separating the complex from NGF by gel filtration.

For the cellular studies, the signal peptide, extracellular and transmembrane domains of human TrkA (nt 1-1344) as well as the full-length rat p75, and a version lacking the p75 intracellular domain (nt 1-861) were amplified by PCR from cDNA. The portion of the EGFR utilized was as previously described (Wehrman et al., 2006). An MfeI site was included in the design of the 5' primers and an XhoI site was included in the 3' primer. The resulting PCR products were cloned into retroviral vectors to produce the N-terminal fusions to the  $\omega$  and YFP- $\alpha$  fragments. The  $\alpha^*$  peptide vector has eYFP cloned as a fusion N-terminal to the peptide (Wehrman et al., 2005).

#### Crystallization

For crystallization, the NGF/TrkA complex was concentrated to 10 mg/ml in HBS. The crystals were made through vapor-diffusion in sitting drops containing equal volumes of protein and the well solution containing 15% PEG20000, 1 M glycine, 0.15 M NaCl, buffered with 0.1 M HEPES (pH 7.5).

#### Data Collection and Processing

Data sets were measured on a  $2 \times 2$  ADSC CCD at Beamline 11.1 at the Stanford Synchrotron Radiation Laboratory (SSRL), Stanford. Crystals were cryoprotected before cooling to 100 K with presence of 20% ethylene glycol in the mother liquor. The data set was collected to 3.4 Å. The crystals have a spacegroup  $P2_1$ , with cell dimensions  $a = 110.22$  Å,  $b = 81.68$  Å,  $c = 115.75$  Å. The data were indexed, integrated, and scaled with HKL2000 (Otwinowski and Minor, 1997).

#### Model Building and Refinement

NGF dimer plus the TrkA-D5 was located by molecular replacement with the structure of previously solved partial complex as a search model. Phases calculated with the partial model were density-modified with CNS (Brunger et al., 1998). Although the partial model is only 45% complete, the high solvent content (73%, Matthews coefficient 4.8) and the 2-fold NCS averaging helped to generate an electron density map in which the rough positions of the LRR domain and the Ig-like domain could be identified. A part of Nogo receptor LRR with the C-terminal cysteine-rich domain and four repeats truncated was then manually placed into the horseshoe-shaped electron density, and the D4 Ig-like domain was placed similarly. Further 2-fold averaging using the mask around the placed domains dramatically improved the quality of the electron density map, and the molecular details of the LRR repeats and the D4  $\beta$  strands emerged, allowing the building of a complete TrkA model.

The structure was refined using CNS (Brunger et al., 1998). Maximum-likelihood simulated annealing using the CNS torsion angle dynamics protocol was carried out. Throughout refinement, all reflection data were used without sigma-cutoff. Repeated iterations between manual rebuilding and minimization as well as B factor refinement finally resulted in a model with converged R factor of 29.7% and  $R_{free}$  of 33.1%. A summary of the refinement statistics and the stereochemistry analysis is given in Table 1.

#### Cell Line Creation

C2C12 myoblasts were obtained from laboratory stocks and grown in DMEM (Invitrogen) 20% FBS (Hyclone) including penicillin and streptomycin. The ecotropic  $\Phi$ NX packaging cell line (P.L. Achacoso and G.P. Nolan, personal communication) was transiently transfected with the plasmid DNA constructs in 6-well dishes using lipofectamine transfection reagent (Invitrogen) according to manufacturer's instructions. The supernatant from the transfected cells was removed 24–72 hr later and applied to C2C12 myoblasts. Polybrene was added to a final concentration of 8  $\mu$ g/ml (Sigma, St. Louis, MO). Transduced cells were selected by FACS on a Becton Dickinson flow cytometer with MoFlo electronics for YFP expression and/or by the addition of geneticin (Invitrogen) at a concentration of 1 mg/ml.

#### $\beta$ -Galactosidase Assays

Carried out essentially as described in Wehrman et al. (2005). Cells were plated at 20,000/well in 100  $\mu$ l volume in white, clear-bottom 96-well plates (Corning Costar, Acton, MA). Media was aspirated and  $\beta$ -galactosidase activity was assayed by addition of GAL-Screen substrate (50  $\mu$ l total volume of a 1:25 solution B to solution A ratio) (Applied Biosystems, buffer B formulation), and the plates were incubated at RT for 40 min. Luminescence was measured in a Tropic TR717 luminometer.

#### Scatchard Analysis

Cells expressing the Trk $\alpha^*$  and p75 $\alpha^*$  were removed from tissue culture dishes using a PBS EDTA mixture. Cells were resuspended at a concentration of  $6 \times 10^6$ /ml in an ice-cold PBS 20% FBS solution.  $^{125}$ I NGF (Amersham) was diluted in a 20% FBS/PBS solution in clear 96-well v-bottom dishes (Corning) on ice. Unlabeled NGF was included where appropriate at  $>1000\times$  concentration of the unlabeled NGF. Cells were added to the NGF solution and incubated on ice for 90 min in a total volume of 60  $\mu$ l, which included 60,000 cells. 200  $\mu$ l of ice-cold FBS was added, and the cells were spun in a refrigerated tabletop Beckman microfuge for 5 min. @ 4,000 RPM. The supernatant was removed with a multichannel pipette and ultimately placed into 5 ml of scintillation fluid for analysis of the "free" fraction. The cell pellet was solubilized with scintillation fluid and placed into 5 ml of scintillation fluid for analysis of the "bound" fraction. Scintillation vials were read on a beckman scintillation counter. The values from the background binding (wells that included  $1000\times$  unlabeled NGF) were subtracted from the bound fraction values prior to Scatchard transformation. Background values averaged  $21\% \pm 12\%$  of the bound values. Scatchard analysis of Trk $\alpha^*$  alone, p75 $\alpha$ -FL\*, and PC12 cells was carried out in identical fashion. PC12 cells were grown in 10% horse serum and 5% FBS on collagen-coated dishes.

#### Supplemental Data

The Supplemental Data for this article can be found online at <http://www.neuron.org/cgi/content/full/53/1/25/DC1/>.

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## REFERENCES

- Arevalo, J.C., Conde, B., Hempstead, B.L., Chao, M.V., Martin-Zanca, D., and Perez, P. (2000). TrkA immunoglobulin-like ligand binding domains inhibit spontaneous activation of the receptor. *Mol. Cell Biol.* *20*, 5908–5916.
- Arevalo, J.C., Conde, B., Hempstead, B.L., Chao, M.V., Martin-Zanca, D., and Perez, P. (2001). A novel mutation within the extracellular domain of TrkA causes constitutive receptor activation. *Oncogene* *20*, 1229–1234.
- Aurikko, J.P., Ruotolo, B.T., Grossmann, J.G., Moncrieffe, M.C., Stephens, E., Leppanen, V.M., Robinson, C.V., Saarna, M., Bradshaw, R.A., and Blundell, T.L. (2005). Characterization of symmetric complexes of nerve growth factor and the ectodomain of the pan-neurotrophin receptor, p75NTR. *J. Biol. Chem.* *280*, 33453–33460.
- Barker, P.A. (2004). p75NTR is positively promiscuous: novel partners and new insights. *Neuron* *42*, 529–533.
- Barker, P.A., and Shooter, E.M. (1994). Disruption of NGF binding to the low affinity neurotrophin receptor p75LNTR reduces NGF binding to TrkA on PC12 cells. *Neuron* *13*, 203–215.
- Battleman, D.S., Geller, A.I., and Chao, M.V. (1993). HSV-1 vector-mediated gene transfer of the human nerve growth factor receptor p75hNGFR defines high-affinity NGF binding. *J. Neurosci.* *13*, 941–951.
- Bibel, M., Hoppe, E., and Barde, Y.A. (1999). Biochemical and functional interactions between the neurotrophin receptors trk and p75NTR. *EMBO J.* *18*, 616–622.
- Bilderback, T.R., Gazula, V.R., and Dobrowsky, R.T. (2001). Phosphoinositide 3-kinase regulates crosstalk between Trk A tyrosine kinase and p75(NTR)-dependent sphingolipid signaling pathways. *J. Neurochem.* *76*, 1540–1551.
- Bothwell, M. (1995). Functional interactions of neurotrophins and neurotrophin receptors. *Annu. Rev. Neurosci.* *18*, 223–253.
- Brunger, A.T., Adams, P.D., Clore, G.M., DeLano, W.L., Gros, P., Grosse-Kunstleve, R.W., Jiang, J.S., Kuszewski, J., Nilges, M., Pannu, N.S., et al. (1998). Crystallography & NMR system: A new software suite for macromolecular structure determination. *Acta Crystallogr. D Biol. Crystallogr.* *54*, 905–921.
- Burke, M.A., and Bothwell, M. (2003). p75 neurotrophin receptor mediates neurotrophin activation of NF- $\kappa$ B and induction of iNOS expression in P19 neurons. *J. Neurobiol.* *55*, 191–203.
- Carter, B.D., Kaltschmidt, C., Kaltschmidt, B., Offenhauser, N., Bohm-Matthaei, R., Baeuerle, P.A., and Barde, Y.A. (1996). Selective activation of NF- $\kappa$ B by nerve growth factor through the neurotrophin receptor p75. *Science* *272*, 542–545.
- Casademunt, E., Carter, B.D., Benzeli, I., Frade, J.M., Dechant, G., and Barde, Y.A. (1999). The zinc finger protein NRIF interacts with the neurotrophin receptor p75(NTR) and participates in programmed cell death. *EMBO J.* *18*, 6050–6061.
- Chang, M.S., Arevalo, J.C., and Chao, M.V. (2004). Ternary complex with Trk, p75, and an ankyrin-rich membrane spanning protein. *J. Neurosci. Res.* *78*, 186–192.
- Chao, M.V. (2003). Neurotrophins and their receptors: a convergence point for many signalling pathways. *Nat. Rev. Neurosci.* *4*, 299–309.
- Chow, D., He, X., Snow, A.L., Rose-John, S., and Garcia, K.C. (2001). Structure of an extracellular gp130 cytokine receptor signaling complex. *Science* *291*, 2150–2155.
- Cohen, S. (1959). Purification and metabolic effects of a nerve growth-promoting protein from snake venom. *J. Biol. Chem.* *234*, 1129–1137.
- Dechant, G., and Barde, Y.A. (2002). The neurotrophin receptor p75(NTR): novel functions and implications for diseases of the nervous system. *Nat. Neurosci.* *5*, 1131–1136.
- Esposito, D., Patel, P., Stephens, R.M., Perez, P., Chao, M.V., Kaplan, D.R., and Hempstead, B.L. (2001). The cytoplasmic and transmembrane domains of the p75 and Trk A receptors regulate high affinity binding to nerve growth factor. *J. Biol. Chem.* *276*, 32687–32695.
- Frade, J.M., Rodriguez-Tebar, A., and Barde, Y.A. (1996). Induction of cell death by endogenous nerve growth factor through its p75 receptor. *Nature* *383*, 166–168.
- Grob, P.M., Berlot, C.H., and Bothwell, M.A. (1983). Affinity labeling and partial purification of nerve growth factor receptors from rat pheochromocytoma and human melanoma cells. *Proc. Natl. Acad. Sci. USA* *80*, 6819–6823.
- Hamanoue, M., Middleton, G., Wyatt, S., Jaffray, E., Hay, R.T., and Davies, A.M. (1999). p75-mediated NF- $\kappa$ B activation enhances the survival response of developing sensory neurons to nerve growth factor. *Mol. Cell. Neurosci.* *14*, 28–40.
- Hantzopoulos, P.A., Suri, C., Glass, D.J., Goldfarb, M.P., and Yancopoulos, G.D. (1994). The low affinity NGF receptor, p75, can collaborate with each of the Trks to potentiate functional responses to the neurotrophins. *Neuron* *13*, 187–201.
- Harrington, A.W., Kim, J.Y., and Yoon, S.O. (2002). Activation of Rac GTPase by p75 is necessary for c-jun N-terminal kinase-mediated apoptosis. *J. Neurosci.* *22*, 156–166.
- He, X.L., and Garcia, K.C. (2004). Structure of nerve growth factor complexed with the shared neurotrophin receptor p75. *Science* *304*, 870–875.
- Hempstead, B.L., Patil, N., Thiel, B., and Chao, M.V. (1990). Deletion of cytoplasmic sequences of the nerve growth factor receptor leads to loss of high affinity ligand binding. *J. Biol. Chem.* *265*, 9595–9598.
- Hempstead, B.L., Martin-Zanca, D., Kaplan, D.R., Parada, L.F., and Chao, M.V. (1991). High-affinity NGF binding requires coexpression of the trk proto-oncogene and the low-affinity NGF receptor. *Nature* *350*, 678–683.
- Howe, C.L., and Mobley, W.C. (2005). Long-distance retrograde neurotrophic signaling. *Curr. Opin. Neurobiol.* *15*, 40–48.
- Huang, E.J., and Reichardt, L.F. (2003). Trk receptors: roles in neuronal signal transduction. *Annu. Rev. Biochem.* *72*, 609–642.
- Huber, L.J., and Chao, M.V. (1995). A potential interaction of p75 and trkA NGF receptors revealed by affinity crosslinking and immunoprecipitation. *J. Neurosci. Res.* *40*, 557–563.
- Ibanez, C.F., Ebendal, T., Barbany, G., Murray-Rust, J., Blundell, T.L., and Persson, H. (1992). Disruption of the low affinity receptor-binding site in NGF allows neuronal survival and differentiation by binding to the trk gene product. *Cell* *69*, 329–341.
- Jing, S., Tapley, P., and Barbacid, M. (1992). Nerve growth factor mediates signal transduction through trk homodimer receptors. *Neuron* *9*, 1067–1079.
- Johnson, D., Lanahan, A., Buck, C.R., Sehgal, A., Morgan, C., Mercer, E., Bothwell, M., and Chao, M. (1986). Expression and structure of the human NGF receptor. *Cell* *47*, 545–554.
- Kalb, R. (2005). The protean actions of neurotrophins and their receptors on the life and death of neurons. *Trends Neurosci.* *28*, 5–11.
- Kaplan, D.R., Hempstead, B.L., Martin-Zanca, D., Chao, M.V., and Parada, L.F. (1991). The trk proto-oncogene product: a signal transducing receptor for nerve growth factor. *Science* *252*, 554–558.
- Karchewski, L.A., Kim, F.A., Johnston, J., McKnight, R.M., and Verge, V.M. (1999). Anatomical evidence supporting the potential for modulation by multiple neurotrophins in the majority of adult lumbar sensory neurons. *J. Comp. Neurol.* *413*, 327–341.
- Khursigara, G., Orlicki, J.R., and Chao, M.V. (1999). Association of the p75 neurotrophin receptor with TRAF6. *J. Biol. Chem.* *274*, 2597–2600.
- Klein, R., Jing, S.Q., Nanduri, V., O'Rourke, E., and Barbacid, M. (1991). The trk proto-oncogene encodes a receptor for nerve growth factor. *Cell* *65*, 189–197.

- Klotz, I.M., and Hunston, D.L. (1984). Mathematical models for ligand-receptor binding. Real sites, ghost sites. *J. Biol. Chem.* 259, 10060–10062.
- Kobe, B., and Deisenhofer, J. (1994). The leucine-rich repeat: a versatile binding motif. *Trends Biochem. Sci.* 19, 415–421.
- Lachyankar, M.B., Condon, P.J., Daou, M.C., De, A.K., Levine, J.B., Obermeier, A., and Ross, A.H. (2003). Novel functional interactions between Trk kinase and p75 neurotrophin receptor in neuroblastoma cells. *J. Neurosci. Res.* 71, 157–172.
- Lad, S.P., Peterson, D.A., Bradshaw, R.A., and Neet, K.E. (2003). Individual and combined effects of TrkA and p75NTR nerve growth factor receptors. A role for the high affinity receptor site. *J. Biol. Chem.* 278, 24808–24817.
- Lee, K.F., Li, E., Huber, L.J., Landis, S.C., Sharpe, A.H., Chao, M.V., and Jaenisch, R. (1992). Targeted mutation of the gene encoding the low affinity NGF receptor p75 leads to deficits in the peripheral sensory nervous system. *Cell* 69, 737–749.
- MacDonald, J.I., and Meakin, S.O. (1996). Deletions in the extracellular domain of rat trkA lead to an altered differentiative phenotype in neurotrophin responsive cells. *Mol. Cell. Neurosci.* 7, 371–390.
- Mahadeo, D., Kaplan, L., Chao, M.V., and Hempstead, B.L. (1994). High affinity nerve growth factor binding displays a faster rate of association than p140trk binding. Implications for multi-subunit polypeptide receptors. *J. Biol. Chem.* 269, 6884–6891.
- Majdan, M., Lachance, C., Gloster, A., Aloyz, R., Zeindler, C., Bamji, S., Bhakar, A., Belliveau, D., Fawcett, J., Miller, F.D., and Barker, P.A. (1997). Transgenic mice expressing the intracellular domain of the p75 neurotrophin receptor undergo neuronal apoptosis. *J. Neurosci.* 17, 6988–6998.
- Mischel, P.S., Smith, S.G., Vining, E.R., Valletta, J.S., Mobley, W.C., and Reichardt, L.F. (2001). The extracellular domain of p75NTR is necessary to inhibit neurotrophin-3 signaling through TrkA. *J. Biol. Chem.* 276, 11294–11301.
- Nekhai, S.A., Beletzkij, V.E., and Graifer, D.M. (1997). Influence of systematic error on the shape of the scatchard plot of tRNAPhe binding to eukaryotic ribosomes. *Biochem. J.* 325, 401–404.
- Nicosia, S. (1988). More about the misuse of Scatchard plots in binding studies. *Pharmacol. Res. Commun.* 20, 733–737.
- Nykjaer, A., Willnow, T.E., and Petersen, C.M. (2005). p75NTR—live or let die. *Curr. Opin. Neurobiol.* 15, 49–57.
- Otwinowski, Z., and Minor, W. (1997). *Methods Enzymol.* 276, 307–326.
- Perez, P., Coll, P.M., Hempstead, B.L., Martin-Zanca, D., and Chao, M.V. (1995). NGF binding to the trk tyrosine kinase receptor requires the extracellular immunoglobulin-like domains. *Mol. Cell. Neurosci.* 6, 97–105.
- Riccio, A., Pierchala, B.A., Ciarallo, C.L., and Ginty, D.D. (1997). An NGF-TrkA-mediated retrograde signal to transcription factor CREB in sympathetic neurons. *Science* 277, 1097–1100.
- Rifkin, J.T., Todd, V.J., Anderson, L.W., and Lefcort, F. (2000). Dynamic expression of neurotrophin receptors during sensory neuron genesis and differentiation. *Dev. Biol.* 227, 465–480.
- Roux, P.P., and Barker, P.A. (2002). Neurotrophin signaling through the p75 neurotrophin receptor. *Prog. Neurobiol.* 67, 203–233.
- Roux, P.P., Bhakar, A.L., Kennedy, T.E., and Barker, P.A. (2001). The p75 neurotrophin receptor activates Akt (protein kinase B) through a phosphatidylinositol 3-kinase-dependent pathway. *J. Biol. Chem.* 276, 23097–23104.
- Teng, K.K., and Hempstead, B.L. (2004). Neurotrophins and their receptors: signaling trios in complex biological systems. *Cell. Mol. Life Sci.* 61, 35–48.
- Urfer, R., Tsoulfas, P., O'Connell, L., Hongo, J.A., Zhao, W., and Presta, L.G. (1998). High resolution mapping of the binding site of TrkA for nerve growth factor and TrkC for neurotrophin-3 on the second immunoglobulin-like domain of the Trk receptors. *J. Biol. Chem.* 273, 5829–5840.
- Verdi, J.M., Birren, S.J., Ibanez, C.F., Persson, H., Kaplan, D.R., Benedetti, M., Chao, M.V., and Anderson, D.J. (1994). p75LNGFR regulates Trk signal transduction and NGF-induced neuronal differentiation in MAH cells. *Neuron* 12, 733–745.
- von Schack, D., Casademunt, E., Schweigreiter, R., Meyer, M., Bibel, M., and Dechant, G. (2001). Complete ablation of the neurotrophin receptor p75NTR causes defects both in the nervous and the vascular system. *Nat. Neurosci.* 4, 977–978.
- Wang, J.J., Rabizadeh, S., Tasinato, A., Sperandio, S., Ye, X., Green, M., Assa-Munt, N., Spencer, D., and Bredesen, D.E. (2000). Dimerization-dependent block of the proapoptotic effect of p75(NTR). *J. Neurosci. Res.* 60, 587–593.
- Wang, X., Bauer, J.H., Li, Y., Shao, Z., Zetoune, F.S., Cattaneo, E., and Vincenz, C. (2001). Characterization of a p75(NTR) apoptotic signaling pathway using a novel cellular model. *J. Biol. Chem.* 276, 33812–33820.
- Watson, F.L., Porcionatto, M.A., Bhattacharyya, A., Stiles, C.D., and Segal, R.A. (1999). TrkA glycosylation regulates receptor localization and activity. *J. Neurobiol.* 39, 323–336.
- Wehrman, T.S., Casipit, C.L., Gewertz, N.M., and Blau, H.M. (2005). Enzymatic detection of protein translocation. *Nat. Methods* 2, 521–527.
- Wehrman, T.S., Raab, W.J., Caspit, C.L., Doyonnes, R., Pomerantz, J.H., and Blau, H.M. (2006). A system for quantifying dynamic protein interactions defines a role for Herceptin in modulating ErbB2 interactions. *Proc. Natl. Acad. Sci. USA* 103, 19063–19068.
- Wiesmann, C., and de Vos, A.M. (2001). Nerve growth factor: structure and function. *Cell. Mol. Life Sci.* 58, 748–759.
- Wiesmann, C., Ultsch, M.H., Bass, S.H., and de Vos, A.M. (1999). Crystal structure of nerve growth factor in complex with the ligand-binding domain of the TrkA receptor. *Nature* 401, 184–188.
- Windisch, J.M., Auer, B., Marksteiner, R., Lang, M.E., and Schneider, R. (1995a). Specific neurotrophin binding to leucine-rich motif peptides of TrkA and TrkB. *FEBS Lett.* 374, 125–129.
- Windisch, J.M., Marksteiner, R., Lang, M.E., Auer, B., and Schneider, R. (1995b). Brain-derived neurotrophic factor, neurotrophin-3, and neurotrophin-4 bind to a single leucine-rich motif of TrkB. *Biochemistry* 34, 11256–11263.
- Wright, D.E., and Snider, W.D. (1995). Neurotrophin receptor mRNA expression defines distinct populations of neurons in rat dorsal root ganglia. *J. Comp. Neurol.* 357, 329–338.
- Yano, H., and Chao, M.V. (2000). Neurotrophin receptor structure and interactions. *Pharm. Acta Helv.* 74, 253–260.
- Ye, X., Mehlen, P., Rabizadeh, S., VanArsdale, T., Zhang, H., Shin, H., Wang, J.J., Leo, E., Zapata, J., Hauser, C.A., et al. (1999). TRAF family proteins interact with the common neurotrophin receptor and modulate apoptosis induction. *J. Biol. Chem.* 274, 30202–30208.
- Yeiser, E.C., Rutkoski, N.J., Naito, A., Inoue, J., and Carter, B.D. (2004). Neurotrophin signaling through the p75 receptor is deficient in traf6<sup>-/-</sup> mice. *J. Neurosci.* 24, 10521–10529.
- Zaccaro, M.C., Ivanisevic, L., Perez, P., Meakin, S.O., and Saragovi, H.U. (2001). p75 Co-receptors regulate ligand-dependent and ligand-independent Trk receptor activation, in part by altering Trk docking subdomains. *J. Biol. Chem.* 276, 31023–31029.
- Zierler, K. (1989). Misuse of nonlinear Scatchard plots. *Trends Biochem. Sci.* 14, 314–317.

#### Accession Numbers

The coordinates and structure factors have been deposited in the Protein Data Bank, with the accession code 2IFG.

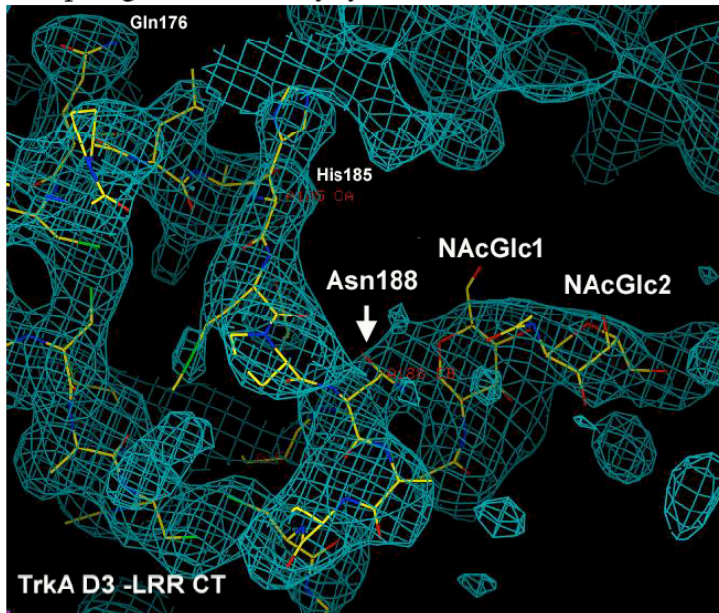
Neuron, Volume 52

**Supplemental Data**

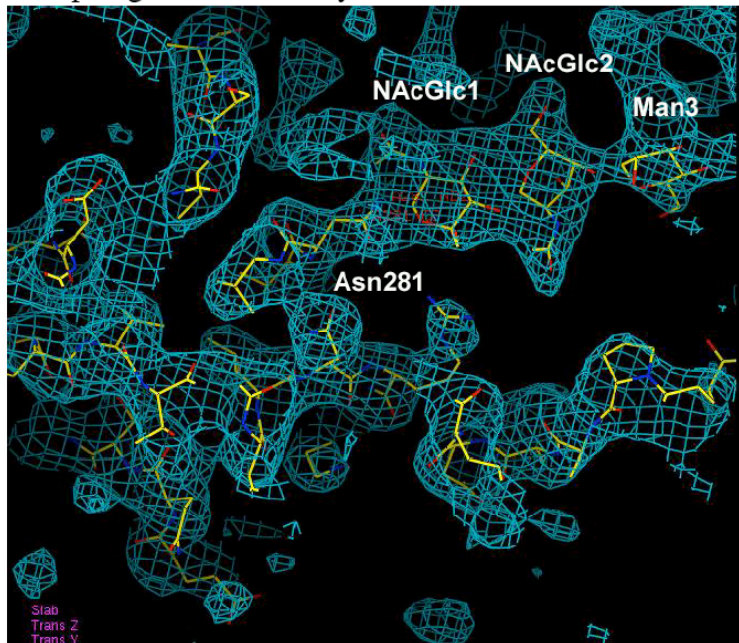
**Structural and Mechanistic Insights into  
Nerve Growth Factor Interactions with the  
TrkA and p75 Receptors**

**Tom Wehrman, Xiaolin He, Bill Raab, Abhiram Dukipatti,  
Helen Blau, and K. Christopher Garcia**

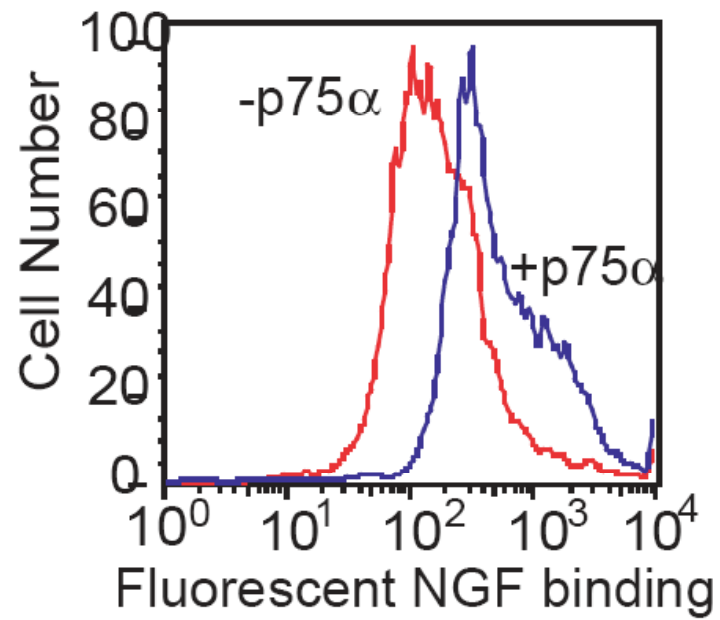
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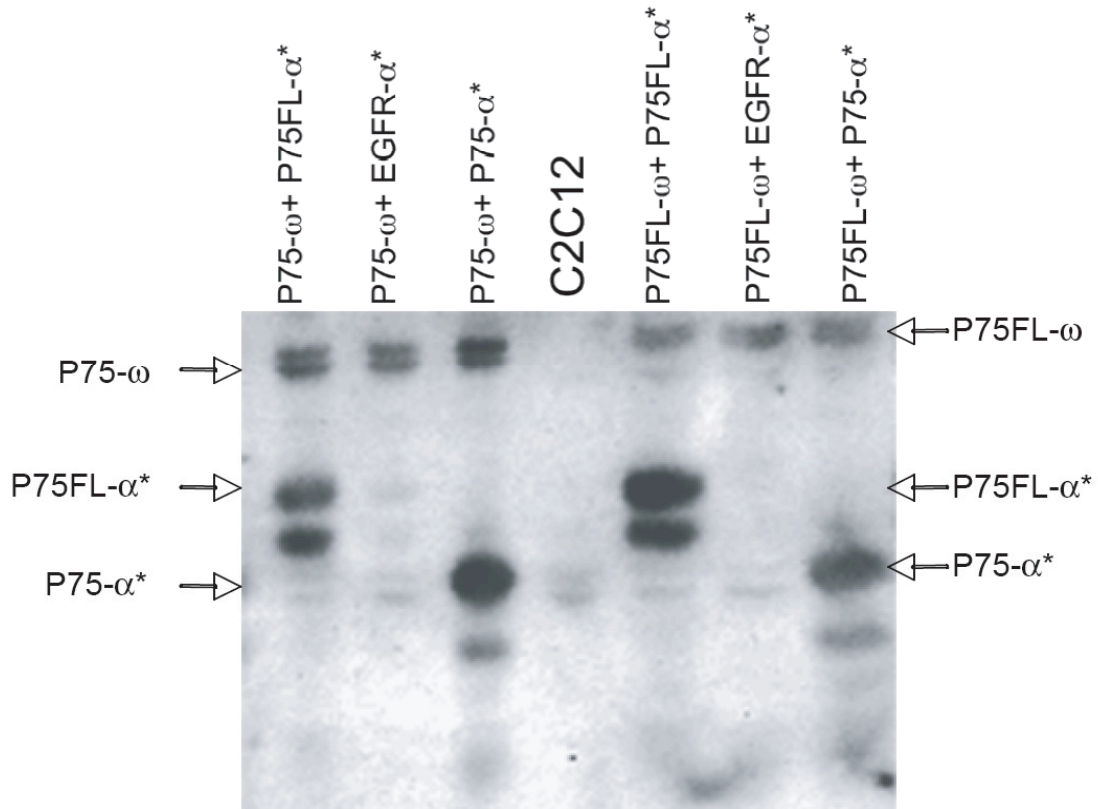


Supplementary Figure 1. Electron density of Asparagine-linked carbohydrates on the TrkA structure. SigmaA 2Fo-Fc maps were calculated without coordinates of the carbohydrate residues, so glycan electron density is unbiased.



Supplementary Figure 2.

The truncated p75 receptor binds NGF. C2C12 cells expressing p75a\* were incubated with 1ug/ml recombinant NGF labeled with Alexa 647 (blue curve). Untransduced C2C12 cells also incubated with the ligand serve as the negative control (red curve)



Supplementary Figure 3.

C2C12 cells do not express significant amounts of endogenous p75. The C2C12 cell lines transfected with the p75 and TrkA beta-gal constructs and used for the beta-gal complementation experiments were probed with anti-p75 antibody by Western blot. As can be seen, only the cells transfected with p75 show bands for either full-length p75 or truncated p75.