TrkA Receptor “Hot Spots” for Binding of NT-3 as a Heterologous Ligand*

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Ljubica Ivanisevic†‡§, WenHua Zheng†, Sang B. Woo†, Kenneth E. Neet¶, and H. Uri Saragovi†§‖‡

From the †Lady Davis Institute-Jewish General Hospital, ‡Department of Pharmacology and Therapeutics, §Oncology/Cancer Center, McGill University, Montreal, Quebec H3T 1E2, Canada and the †Department of Biochemistry and Molecular Biology, Rosalind Franklin University of Medicine and Science, North Chicago, Illinois 60064

Neurotrophins signal via Trk tyrosine kinase receptors. Nerve growth factor (NGF) is the cognate ligand for TrkA, the brain-derived neurotrophic factor for TrkB, and NT-3 for TrkC. NT-3 also binds TrkA as a lower affinity heterologous ligand. Because neurotrophin-3 (NT-3) interactions with TrkA are biologically relevant, we aimed to define the TrkA “hot spot” functional docking sites of NT-3. The Trk extracellular domain consists of two cysteine-rich subdomains (D1 and D3), flanking a leucine-rich subdomain (D2), and two immunoglobulin-like subdomains IgC1(D4) and IgC2(D5). Previously, the D5 subdomain was defined as the primary ligand-binding site of neurotrophins for their cognate receptors (e.g. NGF) binds and activates through TRKA-D5 hot spots). Here binding studies with truncated and chimeric extracellular subdomains show that TRKA-D5 also includes an NT-3 docking and activation hot spot (site 1), and competition studies show that the NGF and NT-3 hot spots on TRKA-D5 are distinct but partially overlapping. In addition, ligand binding studies provide evidence for an NT-3-binding/allosteric site on TRKA-D4 (site 2). NT-3 docking on sites 1 and/or 2 partially blocks NGF binding. Functional survival studies showed that sites 1 and 2 regulate TrkA activation. NT-3 docking on both sites 1 and 2 affords full agonism, which can be additive with NGF activation of Trk. However, NT-3 docking solely on site 1 is partially agonistic but noncompetitively antagonizes NGF binding and activation of Trk. This study demonstrates that Trk signaling is more complex than previously thought because it involves several receptor subdomains and hot spots.

The neurotrophins are a family of growth factors that regulate proliferation, survival, death, differentiation of neurons in the embryonic and early postnatal stages, neuronal maintenance, synaptic activity, and learning later in life (1, 2). Nerve growth factor (NGF),4 neurotrophin-3 (NT-3), neurotrophin-4, and brain-derived neurotrophic factor (BDNF) are members of the neurotrophin family of polypeptides.

Neurotrophins act by binding to two distinct classes of transmembrane receptors. One is the p75 neurotrophin receptor and the other is the Trk family of tyrosine kinase receptors, which includes TrkA, TrkB, and TrkC. All mature neurotrophins bind to p75, but Trks are more selective. NGF interacts selectively with TrkA receptors and BDNF selectively with TrkB receptors. Although NT-3 interacts with TrkC receptors preferably, it is promiscuous and can also bind TrkA and TrkB with lower affinity (3). The Trk receptors mediate most of the “positive” survival and differentiation signals typically associated with neurotrophin activity.

Nevertheless, NT-3-TrkA interactions are biologically relevant. For example, during development NGF and NT-3 act coordinately to select TrkA-expressing sympathetic neurons (4). Furthermore, sympathetic neurons from NGF null and NT-3 null mice die when these neurons express high levels of TrkA and negligible levels of TrkC (5). NT-3 mediates neurogenesis as effectively as NGF, but it affords ~20–40-fold less survival compared with NGF in NGF-dependent sympathetic neurons (6). Also, NGF and NT-3 acting via TrkA are required for sympathetic axon growth and target innervation (7).

The extracellular domain of Trk receptors features five subdomains defined by their homology to other proteins (8). Near the N terminus is a leucine-rich motif (also known as D2), flanked by two cysteine-rich clusters (Cys-1 and Cys-2, also known as D1 and D3, respectively). Closer to the transmembrane region, there are two immunoglobulin-like subdomains termed Ig-C1(D4) and Ig-C2(D5) (Fig. 1). NTFs transduce trophic signals by binding to the extracellular domain of Trk and stabilizing receptor homodimerization (9). It is postulated that a conformational change is induced upon ligand binding, which then activates the intrinsic intracellular tyrosine kinase catalytic activity (10).

Previous work showed that the D5 subdomains of TrkA, TrkB, and TrkC are relevant for binding NGF, BDNF, and NT-3, respectively (11–15), and a recombinant polypeptide spanning TRKA-D4 and D5 (TRKA-D4–D5) bound and neutralized NGF (16). Moreover, it has also been shown that the D4 subdomain has a role in regulating receptor dimerization (17)
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and in constitutive activation of the receptors (18). The D1 subdomain of TrkA can be utilized by NGF to activate the receptor, through a potentially allosteric or conformational mechanism regulated by co-expression of p75 on the cell surface (18).

The fact that NT-3 has a lower affinity interaction with its heterologous receptors TrkA and TrkB (13, 19), and that the biological outcomes of NT-3-TrkA interactions are limited (e.g. partial survival, or limited differentiation) (6) suggests three nonexclusive possibilities as follows: (i) NT-3 as heterologous ligand may lack some docking sites on TrkA for high affinity binding and full function; (ii) the interaction may take place through epitopes that are distinct from those used by NGF; and/or (iii) receptor conformational or allosteric regulation by each ligand may be different.

The goal of our study was to address these possibilities by determining the binding and functional profile of the extracellular domain of TrkA toward cognate NGF or heterologous NT-3 ligands. We report that most of the TrkA activation by cognate or heterologous ligands occurs via the D5 subdomain (site 1). NT-3 binds and activates also at TRKA-D4. Binding of NT-3 to each site seems to be associated with a particular mode of TrkA activation, which can be antagonistic or additive toward NGF. Our study demonstrates that Trk binding and signaling is complex because it involves several receptor subdomains, including potentially allosteric sites that may have positive or negative effects and may be neurotrophin-specific.

MATERIALS AND METHODS

Neurotrophins—Recombinant human NT-3 produced in Escherichia coli was purchased from Prospec-Tany Techno-Gene LTD (Rehovot, Israel). Nerve growth factor isolated from mouse submaxillary gland was purchased from Prince Labs (Toronto, Canada).

Rat TrkA-Rat TrkB Chimeras—The chimeric receptors were constructed by subcloning the rat TRKA-D5 domain sequence into the corresponding unique restriction sites of the rat TRKB cDNA reported in previous work (11). The chimeric construct was confirmed by sequencing and was cloned into the pCDNA3 expression vector that contains the neomycin (Invitrogen) selection marker.

Transfection and Stable Expression in 293 Cells—HEK293 cells (human kidney epithelium, TrkA<sup>−/−</sup>, p75<sup>−/−</sup>) were transfected with the chimeric cDNA construct using the Lipofectamine Plus method (Invitrogen). Quantitative Western blot analysis (20) with a polyclonal antibody directed to the Trk intracellular domain (203 antisera, a gift of David Kaplan, University of Toronto) indicated that the stable HEK293 4.1 TrkA/B cells express 40,000–150,000 chimeric receptors/cell (data not shown) (18). All cells were grown in RPMI 1640 supplemented with 5% fetal calf serum, antibiotics, and glutamine. Selection for the pCDNA3 expression vector was maintained at 0.4 mg/ml neomycin.

Cell Survival Assays—Cell survival was measured by quantitative tetrazolium salt reagent (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Sigma) and optical density readings as described (21). Cells were plated in 96-well plates (BD Biosciences) at ~5,000 cells/well in serum-free media (SFM) (PFHM-II; Invitrogen), with 0.2% bovine serum albumin (BSA) (Roche Applied Science). Wells were then supplemented with SFM (negative control), with the indicated concentration of the indicated neurotrophin in SFM (test), or with 5% serum (positive control) for ~48 h. Cell growth/survival was calculated relative to 2 nM NGF (standardized to 100%). All assays were repeated at least three times, n = 4–6 per assay.

Primary Antibodies—Anti-TrkA mouse mAb 5C3 binds to the TRKA-D5 subdomain with K<sub>d</sub> ~ 2 nM and acts as a full agonist of this receptor (22). Mouse NGF30 mAb is an anti-NGF mAb that when bound to NGF still allows high affinity binding of NGF to TrkA. The interaction of NGF-NGF30 complexes with TrkA affords survival but not differentiation of TrkA-expressing cells (23). NGF-NGF30-FITC complexes are used at 2-fold excess of mAb (20 nM NGF plus 40 nM NGF30 mAb).

FACScan Analysis—HEK293 cells expressing 4.1 TrkA/B receptor or NIH-3T3 cells expressing TrkA WT receptor (2 × 10<sup>5</sup> cells/tube) were resuspended in 50 μl of FACScan buffer (phosphate-buffered saline, 0.5% BSA, and 0.1% NaN<sub>3</sub>). Cells were untreated or treated with competitors for 20 min at 4 °C. The saturating concentrations of primary antibodies were then added without washing and incubated for 20 min at 4 °C. Excess primary antibody was washed off, and cells were immunostained with fluoresceinated goat anti-mouse IgG. For background staining control, nonspecific mouse IgG or NGF30-FITC antibody in the absence of NGF was used. Maximal binding was obtained by staining with mAb 5C3 or NGF-NGF30 complex without preincubation with neurotrophins. Cells were acquired on a FACScan, and bell-shaped histograms were analyzed using the CellQuest program.

Expression of Complete TrkA-Extracellular Domain and TRKA-D4–D5 Domains—Human TrkA-extracellular domain (TrkA-ECD) was produced in baculovirus. TrkA-ECD contains ~25 kDa of carbohydrate modifications and binds NGF with 3 nM affinity (23, 24). The Ig-C1(D4) and Ig-C2(D5) subdomains of human TrkA (TRKA-D4–D5) were produced in yeast.

TRKA-D4–D5 was cloned by digestion of human TRKA cDNA with Bswl-BbsI, and a 644-bp fragment (coding for amino acids 232–444) was isolated (Qiagen, Mississauga, Ontario, Canada). The fragment was blunt-ended and ligated to pPICZaB vector (Invitrogen) that was predigested with Pmel. A tag containing Myc antibodies (Invitrogen) was used to induce expression of Myc sequences and six histidines (Myc-His tag) was used in frame at the C terminus of the protein by double digestion with KpnI-XbaI, followed by blunting and ligation. Clones were selected using Zeocin antibiotic and verified by diagnostic restriction enzyme analysis and sequencing. Pichia yeast strain KM71 were transformed and selected in Zeocin antibiotic. Expression was carried out for 4 days in buffered methanol-complex medium (BMMY medium, 1% yeast extract, 2% peptone, 1.34% yeast nitrogen base, 4 × 10<sup>−5</sup> M biotin, 0.5% methanol). The supernatant was concentrated (Millipore, Bedford, MA), and the secreted His-Myc-TRKA-D4–D5 was purified using Talon resin, which binds the His tag (Clontech). SDS-PAGE and Western blotting of the purified protein using anti-Myc antibodies (Invitrogen) revealed the presence of a single band of ~55 kDa containing the Myc tag. The purity of TRKA-D4–D5 was ascertained by silver staining (Bio-Rad). The pro-
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tein contains ~25 kDa of carbohydrate post-translational modifications.

Quantification of TrkA-ECD and TRKA-D4–D5—Because of the differences in glycosylation and refolding machinery between the baculovirus and the yeast expression systems, it was expected that there could be qualitative differences between TrkA-ECD and TRKA-D4–D5. Therefore, prior to binding studies, we used mAb 5C3 (22) that binds a conformationally sensitive epitope near the NGF-binding site of these recombinant proteins. ELISA studies were done to quantify appropriately folded TrkA-ECD and TRKA-D4–D5 as described previously (23).

\[ ^{125} \text{I}-\text{NGF Binding Studies on HEK293 Cells Expressing 4.1 Chimeric Receptors} \]

—Scatchard plot analysis of \(^{125}\text{I}-\text{NGF}\) binding to cells was done as described (22, 23). Viable HEK293 cells expressing the 4.1 chimera (with TrkB-D5 subdomain replaced by subdomain D5 of TrkA) were used as test, and viable wild type HEK293 cells were used to determine nonspecific \(^{125}\text{I}-\text{NGF}\) binding. Nonspecific binding was always <20% of total binding and was subtracted. All dilutions and washes were done in binding buffer (PBS, 1% BSA, 0.05% sodium azide), and all procedures were carried out on ice. Cells (5 \(\times\) 10\(^\text{6}\) /100 \(\mu\)l) were added to 100-\(\mu\)l serial dilutions of \(^{125}\text{I}-\text{NGF}\) in the absence or presence of a constant ratio of a 250-fold excess of cold NTFs as competitors. Final volumes of 200 \(\mu\)l were incubated for 30 min with occasional shaking. Fractionation of cell-bound and free counts was done in two alternative ways, both yielding comparable data. In one method, we performed a single 1-ml wash and then resuspended each cell pellet (100 \(\mu\)l) onto 1.0 ml of a serum/sucrose gradient in a new tube. Centrifugation through this gradient separates cells (and cell-bound counts/min) from supernatant. The tubes were frozen in dry ice immediately after centrifugation, and while frozen, they were cut to separate cells (bottom pellet) from the supernatant (top layer). All washes and supernatants were collected for counting (free counts/min). In the other method, three 1-ml washes were done in a fresh tube, and supernatants (free) and pellet (bound) were collected and counted.

\[ \text{Biacore Binding Studies} \]

—The rat TrkA-rat TrkB 4.1 ECD chimera was generated by PCR using the full-length rat TrkA-rat TrkB receptor chimeric cDNAs (18) and cloned into pBlueBac4.5/V5-His vector (Invitrogen). The integrity and frame of the constructs were verified by sequencing (data not shown). The human TrkA-ECD or rat chimeric ECD were each overexpressed in SF21 insect cells in XL-401 medium (JRH Biosciences, Lenexa, KA) as described previously (24). Proteins were purified with their C-terminal His-tag by nickel-nitrilotriacetic acid chromatography and subsequent steps to near homogeneity as shown with SDS-polyacrylamide gels stained with Coomassie Blue (24). The human Trk-ECD wild type or rat 4.1 chimera was immobilized on a CM4 sensor chip using EDC/NHS coupling chemistry and analyzed on a Biacore 3000 instrument (Biacore, Piscataway, NJ) using BIA Evaluation software (version 4.0.1, Biacore). For this family, it has been appropriate to compare human and rat receptors, and ligands from either species bind to and activate receptors from either species with no detectable differences.

Trk and Akt Activation—NIH-3T3 TrkA WT or HEK293 4.1 TrkB receptors expressing cells were collected (5 \(\times\) 10\(^\text{5}\)/point), washed with PBS, and incubated in SFM at 37 °C for 30 min to reduce background receptor phosphorylation levels. The kinetics of activation of each protein (TrkA WT or 4.1 TrkB/Akt) was followed after treatment of rested live cells with different concentrations of ligands for 3, 12, or 30 min at 37 °C. Cells were washed in ice-cold PBS and lysed in detergent lysis buffer (1% Nonident P-40, 20 mM Tris, pH 7.5, 137 mM NaCl, 2 mM EDTA, 10 mM benzamidine, 50 mM sodium orthovanadate, 10 mg/ml leupeptin, 2 mg/ml soybean trypsin inhibitor, 1 mM iodoacetamide, and 10 mg/ml aprotinin), and protein concentrations were determined with detergent-compatible protein assay (Bio-Rad). Western blot analysis was performed with anti-phosphotyrosine (Tyr(P)) antibody 4G10 (Upstate Biotechnology, Inc., Lake Placid, NY), anti-phospho-TrkATyr-490 (Cell Signaling), and anti-phospho-Akt (Ser-473) antibody (New England Biolabs). Blots were visualized using the enhanced chemiluminescence system (PerkinElmer Life Sciences). Re-blotting the membranes with anti-actin antibody confirmed equal loading.

ELISA—TrkA-ECD (10 ng/well), yeast TRKA-D4–D5 (50 ng/well), or negative control proteins were immobilized onto 96-well microtest ELISA plates (BD Biosciences), followed by blocking with blocking buffer (PBS with 1% BSA) for 1 h. The indicated concentrations of NT-3 or control proteins were added, and the NGF-NGF30 complex was then added. ELISA was performed as described (23). Representative assay of three independent experiments is shown, n = 4 per assay. Throughout, anti-TrkA mAb 5C3 was used to control the presence of appropriately folded TrkA-ECD or TRKA-D4–D5 on plates.

Statistical Analysis—The data were analyzed by two-tailed Student’s t test, and p values are reported. Significance is p ≤ 0.05 and is indicated in appropriate figures by asterisks.

RESULTS

Chimeras Exhibit Trophic Signals in Response to Neurotrophins—Full-length TrkB receptors expressing the D5 (lg-C2) subdomain of rat TrkA were generated, and the resulting chimera was termed 4.1 (18). The 4.1 TrkB/A chimeric receptor cDNA and TrkA wild type receptor cDNA were stably transfected in HEK293 and NIH cells. The structure and domain organization of TrkA WT and 4.1 TrkB/A chimeric receptor are illustrated in Fig. 1A.

Cells cultured in SFM undergo apoptotic death, which can be rescued by neurotrophins if the cells express functional neurotrophin receptors. We have confirmed (data not shown) the differential effect of NT-3 and NGF with various receptor constructs (18). In wild type TrkA-expressing cells, 2 nM NGF affords optimal survival. NT-3 also protects wild type TrkA receptors expressing cells but with lower potency. NT-3 at 2 nM protects ~20% and at 500 nM protects ~100%. In cells expressing the 4.1 TrkB chimera, 2 nM NGF affords optimal survival of 100%. NT-3 protects 4.1 TrkB chimera-expressing cells but to a significantly lesser degree. NT-3 at 2 nM protects ~20% and at 500 nM protects ~30%. Hence, NGF activates fully via the

\[ ^{5} \text{S. B. Woo, T. Fritz, J. Page, H. U. Sargovi, and K. E. Neet, manuscript in preparation.} \]
TRKA-D5 subdomain, whereas NT-3 activates only partially via the TRKA-D5 subdomain or via the TrkB-D1–D4 subdomains of the chimera. To understand the relationship between NT-3 and NGF-docking sites on TrkA, we carried out direct ligand binding on purified proteins comprising either the 4.1 TrkA/B ECD or the whole TrkA extracellular domain.

Direct Ligand-Receptor Binding Studies—Biacore surface plasmon resonance was used to obtain $k_{on}$ and $k_{off}$ values for NGF and NT-3 binding to TrkA-ECD or TrkA/B 4.1 chimera ECD and to obtain $K_d$ values for these ligands. NGF binds to TrkA-ECD with $K_d = 1.91 \pm 0.03$ nM. On the other hand, NT-3 binds to TrkA-ECD with $K_d = 131 \pm 22$ nM. The on rates were similar, but the $k_{off}$ was significantly higher for NT-3, leading to the resultant higher $K_d$ (Table 1). The Biacore data for NGF binding to 4.1 TrkA/B chimera ECD shows $K_d = 292 \pm 6$ pm, but NT-3 binding to this chimERIC protein was beyond the limit of detection ($>300$ nM), and it was not possible to determine a $K_d$ value. Thus, the main subdomain for NGF and NT-3 binding is TrkA D5, but other subdomains affect the affinity or the kinetics.

NT-3 Competition of NGF-Receptor Binding—Next, we performed Scatchard analysis of $^{125}$I-NGF binding to 4.1 TrkA/B-expressing HEK293 cells to further assess NT-3 and NGF interactions at the D5 subdomain (Fig. 2). We previously demonstrated high affinity $^{125}$I-NGF binding to 4.1 TrkA/B chimeric receptor expressing cells. This clone expresses ~50,000 chimeric receptors/cell for specific binding with a $K_d = 80 \pm 30$ pm for NGF (18). All of the binding sites were blocked by excess unlabeled NGF. Nonspecific control proteins did not affect $^{125}$I-NGF binding (data not shown).

In cells expressing chimeric 4.1 receptors, addition of 250-fold excess of unlabeled NT-3 reduced the NGF-binding sites in the 4.1 chimera by 30–50%, without substantially affecting the NGF binding affinity of remaining sites (Fig. 2). Higher concentrations of NT-3 did not increase the degree of competition (data not shown). Hence, the interaction of NT-3 with the 4.1 chimera protein is of low affinity and noncompetitive toward NGF. On the other hand, the binding of NT-3 to wild type TrkA is between 10- and 60-fold lower than NGF (our data and see Ref. 19). Direct binding studies with $^{125}$I-NT-3 could not be carried out because this labeled ligand was unavailable to us.

Overall, these data suggest that there are NT-3-binding or allosteric sites on TrkA in subdomains other than TRKA-D5. However, these data cannot discriminate whether NT-3 blocking of NGF is because of steric hindrance (binding to an overlapping site of TRKA-D5) or because NT-3 induces receptor conformational changes.

NGF and NT-3 Bind to the D5 Subdomain of TrkA—A quantitative FACSscan assay and analysis measured competition between TrkA ligands, using TrkA WT receptors expressed on transfected NIH-3T3 cells. The binding of anti-TrkA mAb 5C3, an agonistic TrkA ligand that binds at the D5 subdomain and saturates TrkA at 65 nM (22), was blocked by NGF and NT-3 to different levels. NGF at 50 nM inhibited ~20% of 5C3 binding (Fig. 3, A and C). NT-3 at 50 nM did not compete with 5C3 binding, but 500 nM NT-3 inhibited 5C3 binding by ~20% (Fig. 3, B and D). These results further suggest that both NGF and NT-3 have binding sites in TRKA-D5. However, to achieve the same degree of NGF inhibition of 5C3 binding, it is necessary to use 10-fold higher concentrations of NT-3, suggesting lower affinity binding of NT-3 to TRKA-D5, a different epitope, or differential hindrance.

Possible NT-3 Binding to TrkA D4 Subdomain—To define further the NT-3-binding site on TrkA, we used another TrkA ligand, namely the NGF-NGF30 complex. NGF-

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**Table 1**

Summary of binding data

| NT-3 competition of ligand binding | Purified proteins | Cell lines |
|-----------------------------------|------------------|-----------|
|                                   | TrkA-ECD         | TrkA-D4–D5| TrkA WT   | 4.1 TrkA/B |
| NGF                               | Not done         | Not done  | 40        | 100        |
| NGF-NGF30                         | 20               | 20        | 50        | >500       |

| Biacore direct ligand binding     | TrkA-ECD         | 4.1 TrkA/B ECD | TrkA-ECD | 4.1 TrkA/B ECD |
|-----------------------------------|------------------|----------------|-----------|----------------|
| $K_d$ [nM]                        | 1.91 ± 0.03      | 0.29 ± 0.006   | 131 ± 22  | >300           |
| $k_{on}$ [$10^6$]                 | 0.99 ± 0.002     | 1.78 ± 0.02    | 0.67 ± 0.06| ND             |
| $k_{off}$ [$10^3$]                | 1.9 ± 0.03       | 0.52 ± 0.001   | 87.3 ± 12.5| ND             |

**IC$_{50}$ (nM)**

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NGF30 complex is composed of NGF bound by NGF30 anti-NGF mAb. The complex has different binding and receptor activation profile than free NGF. NGF-NGF30 binds to TrkA with high affinity, but it does not bind p75. The complex induces cell survival but not differentiation (23).

We first tested the binding of the NGF-NGF30 complex to TrkA-ECD and TRKA-D4–D5 purified proteins by ELISA, and we then tested whether NT-3 competes with NGF-NGF30 binding. NGF-NGF30 complex bound to both purified TrkA-ECD and to TRKA-D4–D5. NT-3 competed NGF-NGF30 binding to TrkA-ECD (Fig. 4A) and TRKA-D4–D5 (Fig. 4B) in a dose-dependent manner and with similar profiles. Both receptor fragments were competed equally well by NT-3 from binding NGF-NGF30. NT-3 2 nM blocked ~30% binding to both receptor fragments and NT-3 20 nM blocked ~45% binding to both receptor fragments.

Together with the previous results, these data suggest that NT-3 has a second binding site on TRKA-D4. To confirm the role of TRKA-D4 in NT-3 binding, we tested on receptor-expressing cells the direct binding of the NGF-NGF30 complex to NIH-3T3 TrkA WT and HEK293 4.1 TrkA/B chimeras and competition by NT-3. The NGF-NGF30 complex binds equally well to TrkA WT and 4.1 TrkA/B chimeras. However, whereas NGF-NGF30 binding to WT TrkA was competed efficiently by 50 nM NT-3 (Fig. 4C), similar competition on the 4.1 TrkA/B chimera required more than 25-fold greater NT-3 (Fig. 4E).
Efficient NT-3 competition of NGF-NGF30 binding to TrkA-ECD correlates well with competition of binding to TrkA-WT cells. However, poor NT-3 competition of NGF-NGF30 binding to 4.1 TrkA/B chimeras differs from the efficient competition of TRKA-D4–D5 by ELISA. Differential blocking of 4.1 TrkA/B chimeras (containing only TRKA-D5) and TRKA-D4–D5 further suggests a role of TRKA-D4 in NT-3 binding that cannot be compensated by TrkB-D4 in the chimeric protein.

TrkA Subdomains Direct whether NT-3 Is a Potentiator or an Antagonist of NGF—To determine whether NT-3 binding induces receptor activation, we compared functional responses in survival assays of 4.1 TrkA/B chimera versus TrkA WT-expressing cells. Survival is optimally induced for both cell lines by 2 nM NGF (100%), and 500 nM NGF does not afford significantly higher survival to either cell line. In contrast, 500 nM NT-3 affords 100% survival for TrkA WT cells but only 33% survival for 4.1 TrkA/B (Fig. 5A). Suboptimal but significant survival is
induced by 2 nM NT-3 (~20% for both cell lines) or by 100 pM NGF (~40% for both cell lines), and no survival is induced by 100 pM NT-3 (Fig. 5A). These results are consistent with the estimated affinity of each ligand. Increasing NGF or NT-3 concentrations does not increase survival (data not shown).

Next, we combined NGF and NT-3 as trophic ligands in survival assays. When a constant concentration of 2 nM NGF is applied together with different concentrations of NT-3 (100 pM, 2 nM, and 500 nM), the survival of 4.1 TrkA/B chimera is significantly decreased to 36, 40, and 72%, respectively (p ≤ 0.05), with respect to 2 nM NGF (Fig. 5B). This indicates that NT-3 functionally antagonizes optimal NGF survival activity in the 4.1 TrkA/B chimera. The functional antagonism is robust because approximately half of the remaining survival may be due to the intrinsic NT-3 signals (Fig. 5A). Moreover, it is notable that functional antagonism by NT-3 takes place in the absence of binding antagonism, because 100 pM NT-3 does not block the binding of 2 nM NGF.

In contrast, the survival/growth of TrkA WT cells induced by 2 nM NGF is increased by combining it with NT-3. NGF 2 nM plus NT-3 100 pM affords 116%, NGF 2 nM plus NT-3 2 nM affords 121%, and NGF 2 nM plus NT-3 500 nM affords 160% survival, which is a significant increase (p ≤ 0.05) over 2 nM NGF alone (Fig. 5B). Combinations of low dose NGF and low dose NT-3 (where each alone do not activate) do not afford survival, indicating that there is no synergism (data not shown). These data suggest that NT-3 can activate via receptor sites overlapping with but distinct from the NGF sites, possibly including a site at D4. Thus, the NT-3-binding site on D5 affords partial agonism, whereas NT-3-binding sites on D5 and D4 afford full agonism. See Table 2 for a summary of survival data.

These results suggest that NT-3 acting in 4.1 TrkA/B chimera via TRKA-D5 is a pure partial agonist that can antagonize NGF. In contrast, NT-3 acting in wild type TrkA via D5 and other subdomains (likely D4) can be a full agonist that can be additive with NGF function. It would have been useful to study cellular differentiation in addition to survival, but HEK293 cells do not differentiate in response to NTFs. We have a PC12 variant expressing the 4.1 TrkA/B, but unfortunately it co-expresses p75, which can affect the binding and functional outcome (25); therefore differentiation was not studied.

**Biochemical Correlation of Function**—Western blots using anti-phosphotyrosine antibodies after 12 min of exposure to ligand(s) were used to gauge receptor activation. 2 nM NGF induces strong and comparable phosphorylation of TrkA and 4.1 TrkA/B receptors (Fig. 6, lanes 5 and 13). In contrast, 500 nM NT-3 induces strong phospho-TrkA but very weak phospho-4.1 TrkA/B (Fig. 6, lanes 3 and 11). In five independent experiments, the maximal phospho-4.1 TrkA/B induced by NT-3 was...
Quantification showed that 2 nM NGF plus 500 nM NT-3 affords 54% ± 2 of 4.1 TrkA/B phosphorylation compared versus 2 nM NGF.

To further correlate biochemical data with survival in 4.1 TrkA/B cells, we also studied the kinetics of ligand-dependent activation of the pro-survival PI-3 kinase/Akt pathway and receptor phosphorylation with anti-phospho-Tyr-490 (which recognizes the activated Shc-binding site of Trk). NGF induces rapid and sustained 4.1 TrkA/B phospho-Tyr-490 (Fig. 6, lanes 18–20), but the combination of NGF plus NT-3 induces a transient increased phospho-Tyr-490 at 3 min that decreases to an intermediate level by 12 min (Fig. 6, lanes 24–26), whereas NT-3 alone does not induce a strong 4.1 TrkA/B phospho-Tyr-490 (Fig. 6, lanes 21–23).

Similar data were also obtained for Akt activation, which is known to induce cell survival. NGF causes sustained Akt activation in cells expressing 4.1 chimera, but the combination of NGF plus NT-3 induces lower Akt activation (reduced to ~50% at 3 and 12 min), and the phospho-AKT levels are more comparable with NT-3 than with NGF.

Thus, Trk total phosphorylation, phospho-Tyr-490, and p-Akt correlate well with the survival response of TrkA WT or 4.1 TrkA/B-expressing cells to each ligand or ligand combination. Similar results were obtained when phospholipase Cγ activation was studied (data not shown). These results confirm the notion that NT-3 acting in 4.1 TrkA/B chimera via TrkA-D5 is a pure partial agonist that can antagonize NGF. In contrast, NT-3 acting in wild type TrkA via D5 and other subdomains (likely D4) can be a full agonist that can be additive with NGF function.

**DISCUSSION**

NT-3 and NGF signaling via TrkA control sympathetic neuronal development and differentially regulate survival and differentiation (6, 7). Therefore, we aimed to determine TrkA subdomains involved in NGF and NT-3 binding and the functional outcome of NGF and NT-3 binding to their "hot spots" epitopes on TrkA receptors. NGF-TrkA and NT-3-TrkC activation primarily occurs via the corresponding D5 subdomains of Trks (11). However, potentially allosteric regulation of binding and activity has been shown for Trks, particularly with respect to p75 co-expression (18). Also, p75 on the cell surface affects the affinity of NT-3 for TrkA (25). Because of the potentially confounding factor of p75 co-expression, our studies focused on defining subdomains of TrkA, which are relevant in binding and function toward cognate (NGF) or heterologous (NT-3) ligands in cells and systems that do not contain p75.

We have used different binding methods and functional assays to compare NT-3 and NGF interactions with WT TrkA and the 4.1 Trk-A/B chimera. The $K_d$, $E_C^{50}$, and $I_C^{50}$ values from these methods are summarized in Tables 1 and 2. We determined that NT-3-TrkA binding and signaling involves two receptor subdomains, TRKA-D5 (site 1) and TRKA-D4 (site 2). Furthermore, interaction with both sites is necessary for full NT-3 agonistic activity.

**Binding Studies**—Biacore binding studies showed that the $K_d$ value of TrkA-ECD for NGF (1.9 nM) differs from the 4.1 TrkA/B chimeric ECD (300 pm). The presence of high affinity...
sites was also evident in Scatchard analysis of $^{125}$I-NGF binding studies using HEK293 cells expressing the 4.1 receptor chimera ($K_d\sim80\ pM$). The high affinity binding ($80\ pM$) and curvature apparent in these specific binding data are probably because of the dimerization equilibrium of the Trk receptor under conditions where the receptor is overexpressed at 50,000 receptors per cell.

Higher affinity for the 4.1 TrkA/B chimera suggests that elements in the D1–D4 of TrkA-ECD may be inhibitory of NGF binding to TrkA. Such elements may be within the D1–D3 subdomains of TrkA, by logical exclusion, because the $K_d$ values of TRKA-D4–D5 (16) and TRkA-ECD (24) are similar and independently reported to be $\sim4\ nM$. Previous literature and our data indicate that both NGF and NT-3 bind mainly to the TRKA-D5 subdomain (site 1). However, binding studies showed that NT-3 has relatively lower affinity than NGF for TrkA-ECD (131 nM versus 2 nM), and binding of NT-3 to 4.1 TrkA/B chimeric ECD was undetectable (with a limit of detection $\sim300\ nM$). Therefore, it seems that a second binding site on TrkA D1–D4 may exist for NT-3. NT-3 competition of NGF-NGF30 binding to TrkA-ECD and TRKA-D4–D5 purified proteins indicate that potentially the second NT-3-binding site (site 2) may be allosteric on TRKA-D4. Consistent with this notion, competition of NGF-NGF30 binding to TrkA WT and 4.1 TrkA/B expressed on cells further confirms a role of TRKA-D4 as a potentially allosteric NT-3-binding site. These data also show that TrkB-D4 (within the 4.1 chimeric protein) does not compensate for the contribution of TRKA-D4. In view of this, the D4 subdomains of TrkA and TrkB seem to be different at regulating NT-3 interactions. Indeed, TrkB-D4–D5 subdomains functionally interact with NT-3, because when these domains of TrkB are exchanged for TrkA NT-3 responses are reduced compared with wild type TrkB (18). Based on these results we cannot exclude the possibility that NT-3 interacts with the D4 subdomain of TrkB.

The $IC_{50}$ of NT-3 competition of $^{125}$I-NGF binding requires a 250-fold excess of NT-3 to block binding to 4.1 TrkA/B receptors, whereas a 25-fold excess of NT-3 is required to block binding to wild type TrkA receptors (19). These results further confirm the role of TRKA-D5 subdomain in NT-3 binding, and the 10-fold difference in NT-3 binding to TrkA WT and 4.1 TrkA/B chimera indicates that TrkB-D1–D4 subdomains do not compensate for the equivalent subdomains of TrkA. Taken together, our binding studies define two NT-3-binding sites on the TrkA receptor, one on TRKA-D5 subdomain (site 1) and one on TRKA-D4 subdomain (site 2).

Are the NT-3 and NGF-binding sites on site 1(D5) the same epitope? Competition of 5C3 binding to TrkA WT receptor by NGF or NT-3 points to the distinct but partially overlapping sites for NGF and NT-3 binding on the TRKA-D5 subdomain. This notion is also supported by the poor competition of NT-3 of NGF-NGF30 complexes binding to 4.1 TrkA/B receptors, whereas in TrkA WT receptors competition is very efficient and almost stoichiometric. Different epitopes for these ligands on D5 might explain the functional outcomes of receptor engagement by each ligand (see below).

**TRKA-NT-3 Functional Interactions**—To test if the NT-3 binding to the TrkA receptor induces functional receptor activation, we investigated the survival responses. Both TrkA WT and 4.1 TrkA/B receptor-expressing cells respond equally to NGF in a dose-dependent manner in survival assays. However, their survival response to optimal NT-3 differs, with TrkA WT cells surviving at optimal levels, whereas 4.1 TrkA/B cells exhibit only $\sim30\%$ survival. These results indicate that NT-3 binding to TRKA-D5 subdomain (site 1) induces survival, but TRKA-D4 subdomain (site 2) is necessary for a full survival response.

We also studied phosphorylation of the TrkA or 4.1 TrkA/B receptors and the pro-survival PI-3 kinase/Akt pathway upon Trk receptor activation (26) to correlate long term functional survival assays with short term biochemical signals. The biochemical data confirm that site 1 activation induces survival, acting via the PI-3 kinase/Akt pathway.

In TrkA WT-expressing cells, mixing optimal concentrations of NGF with NT-3 leads to increased cell survival at all the NT-3 concentrations tested. In marked contrast, in 4.1 TrkA/B-expressing cells, mixing optimal NGF with NT-3 leads to decreased survival. The long term functional survival assays are supported by the short term biochemical data.

**Putative Conformational/Allosteric Regulation**—These results require an explanation that reconciles the following data: (a) NT-3 antagonism of NGF binding in all TrkA receptor forms; (b) NT-3 fully activates TrkA WT, but only affords partial activation of 4.1 TrkA/B; (c) NT-3 is functionally antagonistic for NGF in 4.1 TrkA/B, but it enhances TrkA WT receptor function activated by NGF.

How can NT-3 antagonize NGF binding to TRKA-D5, yet enhance NGF activity? One interpretation is that NT-3 activates and is additive with NGF through other TrkA hot spots besides D5. Possibly each of the ligands induces stepwise conformational changes, by binding to each receptor hot spot. NT-3 binding to TRKA-D4 could sterically inhibit NGF or it could cause a receptor conformational change that leads to reduced binding by NGF to D5. Nevertheless, the NGF that does bind can fully activate the receptor. This interpretation further suggests that a conformational change induced by NT-3 binding to TRKA-D4 may have a positive influence in receptor activation.

In view of a recent paper (27), the crystal structure of the entire TrkA-ECD in complex with NGF confirms the primary importance of the D5 subdomain but also indicates lack of flexibility in the TRKA-D1–D4 subdomains. However, the crystal structure of TrkA-ECD alone was not determined; therefore, conformational changes of receptor upon ligand binding cannot be ruled out. The interaction between NT-3 and TrkA-ECD might be different and affect differently the receptor conformation. Moreover, it is interesting that the $K_d$ value for NGF binding to 4.1 chimera is 6-fold stronger than for WT TrkA-ECD, suggesting some inhibitory influence of subdomains D1–D4 upon D5 under some circumstances. This observation may support an allosteric hypothesis. A crystal structure of NT-3-TrkA-ECD would be of considerable interest.

This mechanism would be consistent with other receptor families that have allosteric receptor-binding sites, including human growth hormone (28), erythropoietin receptor (29), and epidermal growth factor receptor (30). However, a comparison
of TRKA-D5 structure (31), deduced from a β-strand swapped D5 dimer, versus a complex of NGF-TRKA-D5 (32) failed to demonstrate conformational changes in either the ligand or the receptor, other than a coiling of a short α-helix in the N terminus of NGF. This rigidity seems quite unusual because generally ligand conformation is affected upon binding and NGF undergoes conformational changes upon binding to p75-ECD (33). Along these lines, CD spectra of TrkA-ECD in complex with NGF showed small but significant conformational changes in secondary structure (24).

NGF and NT-3 signalings via TrkA are important mediators of neuronal development and function, and abnormal signaling via neurotrophin receptors is involved in different pathological states. Identifying the NT-3-binding hot spots on the TrkA receptor will enable synthesis of selective small molecules targeting the receptor-binding or regulatory sites (34), providing further insights into the intricate neurotrophin receptor signaling pathways.

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