p75 Co-receptors Regulate Ligand-dependent and Ligand-independent Trk Receptor Activation, in Part by Altering Trk Docking Subdomains*

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The neurotrophins (NTFs)¹ regulate the survival, death, or differentiation of neurons in the embryonic and early postnatal stages and neuronal maintenance later in life (1). NTFs include nerve growth factor (NGF), neurotrophin-3 (NT-3), and brain-derived neurotrophic factor (BDNF). NGF interacts selectively with TrkA receptors, and BDNF interacts selectively with TrkB receptors. NT-3 interacts with TrkC receptors preferably, but it is more promiscuous and can also bind TrkA and TrkB (2). All NTFs also bind a shared receptor termed p75 with relative low affinity.

NTFs bind to the extracellular domain of Trk and induce receptor homodimerization, leading to activation of the intrinsic intracellular tyrosine kinase catalytic activity (3, 4). The extracellular domain of Trk receptors features five subdomains defined by their homology to other proteins (5). Near the N terminus, there is a leucine-rich motif (LRM), flanked by two cysteine-rich clusters (Cys-1 and Cys-2). Closer to the transmembrane spanning region, there are two immunoglobulin-like subdomains termed IgG-C1 and IgG-C2 (Fig. 1).

Previous work addressed the Trk extracellular domains responsible for NTF binding. Expression of truncated or chimeric receptors in cells showed that IgG-C2 subdomains of TrkA, TrkB, and TrkC are relevant for binding NGF, BDNF, and NT-3, respectively (6–9), and a recombinant polypeptide spanning TrkA IgG-C1 and IgG-C2 bound and neutralized NGF (10). The LRM subdomain was also proposed as ligand binding (11–13); however, expression of LRM-deleted TrkA did not affect NGF binding or activity but altered NT-3 activity (8).

Our study had three aims. The first was to clarify the general architecture of Trk and to define possible "hinge" or regulatory regions that may control receptor activation. While tyrosine kinases undergo conformational changes upon ligand binding (14), analyses failed to demonstrate these changes in Trk (15) or in NGF-IgGC2 crystals (16). Second, we wanted to address the hypothesis that p75 co-expression may allow certain ligands to activate Trks via different extracellular subdomains. This would be expected because there are reports of p75-Trk allosteric or cooperative interactions (17–22). The third aim was to define subdomains of TrkA and TrkB that are functional toward heterologous ligands. While other studies addressed Trk interactions with cognate ligands, studies of heterologous receptor activation by NT-3 were not done.

We find that most of the TrkA or TrkB activation by cognate ligands occurs via the IgG-C2 subdomain. NT-3-mediated activation of TrkA or TrkB is also mediated by the IgG-C2 subdomain but requires additional subdomain(s), probably the IgG-C1. Expression of p75 allows NGF activation of TrkA via its Cys-1-rich subdomain, while BDNF activation of TrkB also involves the LRM and cysteine 2 subdomains when p75 is expressed. The IgG-C1 subdomain is highly exposed in the tertiary structure and seems to control ligand-independent activation. We propose that the IgG-C1 subdomain is a hinge region with intrinsic regulation of receptor conformation and activation. Our data point to a complex, multistage process of regulating receptor-ligand interactions that include intrinsic Trk-regulatory subdomains and receptor-co-receptor interactions.

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MATERIALS AND METHODS

Human TrkA-Rat TrkB Chimeras—Single subdomains (leucine repeats; IgG-C1 and IgG-C2) of human TrkA were generated by polymerase chain reaction using as template a 2.7-kb cDNA encoding the human trkA in Bluescript KS+ vector. Oligonucleotide primers used for the polymerase chain reaction amplification of the human TrkA extra-cellular subdomains: Cys cluster forward, 5’-ATAT GAATTC GCG CAC ATG TCG GGG GAG-3’; Cys cluster backward, 5’-ATAT GTCG GAA ATG AGA-3’. Primer sequences are shown in Fig. 1. The 2.7-kb cDNA construct in each cell line. 

Transfection and Stable Expression in 293 Cells and nnr5 Cells—HEK293 cells (human kidney epithelium, Trk-, p75-) and nnr5 cells (derived from PC12 cells, Trk+, p75++) were transfected with the chimeric cDNA constructs using the LipofectAMINE Plus method (Life Technologies, Inc.). At least three independent subclones of neomycin-resistant cells were obtained by limiting dilution techniques. Quantitative Western blot analysis (23) with a polyclonal antibody directed to the Trk intracellular domain (203 antisera; a gift of David Kaplan, McGill University) indicated that the stable HEK293 subclones express 40,000–100,000 chimeric receptors/cell and that the stable nnr5 subclones express 2,000–6,000 chimeric receptors/cell (data not shown). All cells were grown in RPMI 1640 supplemented with 5% fetal calf serum, antibiotics, and glutamine. Transfectants had 0.4 mg/ml neomycin.

Adenoviral Infection and Expression of p75 in HEK293 Cells—Adenoviruses expressing full-length rat p75 were a kind gift of Sung Ok Yoon (Ohio State University). Expression of p75 in HEK293 cells was achieved by infection at a multiplicity of infection of 10. This resulted in undetectable cell death after 16 h at the time when cells were used for photophosphorylation (Tyr(P)) assays. At 16 h postinfection, >90% of the HEK293 cells had homogeneous expression of p75. Quantitative FAC-Scan assays and Western blotting (24) resulted in an estimate of ~30,000 p75/cell (data not shown), relative to 4-3.6 cells expressing a known density of p75 (21). At longer times postinfection (30 h and above) there was progressive cell death due to viral replication, which precluded us from performing long term cell survival assays.

Cell Survival Assays—Cells used for survival assays were wild type or transfected HEK293 and nnr5. Cell survival was measured by quantitatively tetrazolium salt reagent (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Sigma) and OD readings as described (21, 24). Cells were plated in serum-free media (SFM) (PFHM-II, Life Technologies, Inc.), with 0.5% bovine serum albumin (Roche Molecular Biochemicals), supplemented with SFM (negative control), with 2 nm of the indicated neurotrophin (NTF) (test), or with 5% serum (positive control) for ~68 h. Cell growth/survival was calculated relative to 5% serum (standardized to 100%) to eliminate the possible confounding factor of different growth rates by each clone, as described (21, 24). The difference between 5% serum and SFM supplemented with neurotrophin is that in the former cells survive and grow but in the latter there is poor growth. All assays were repeated with at least three subclones for each construct, and each assay was repeated at least three times (n = 4–6 per assay). Statistical analysis was done by paired student t test with Bonferroni corrections, and significance was p < 0.02.

Western Blotting—Western blots were performed as described (21, 24). Blots were visualized using the enhanced chemiluminescence system (PerkinElmer Life Sciences). For anti-Trk immunoblotting, cells were solubilized, and protein concentrations were determined. Samples were fractionated by SDS-PAGE, transferred to membranes, and immunoblotted with RTA serum (25) directed to the whole extracellular domain of TrkA. Equal protein loading was confirmed by Coomassie Blue staining of gels and by stripping and reblotting membranes with anti-Trk 203 antisera.

Tyrosine Phosphorylation Assays—Cells were washed and rested in SFM at 37°C for 30 min. After ligand treatment (2 nm neurotrophin at 37°C for 12 min), cells were solubilized, and protein concentrations were determined. Samples were fractionated by SDS-polyacrylamide gel electrophoresis, transferred to membranes, and immunoblotted with anti-phosphotyrosine (α-Tyr(P)) antibody 4G10 (Upstate Biotechnology, Inc., Lake Placid, NY). Equal loading was confirmed by stripping and reblotting membranes with anti-Trk 203 antisera.

RESULTS

Full-length TrkB receptors expressing subdomains of human TrkA were generated as illustrated in Fig. 1, using the primers described under “Materials and Methods.” The five chimeric cDNAs and wild type receptor controls were transfected into HEK293 or nnr5 cells, and at least three stable independent clones were obtained for each construct. All biological data were reproduced with three independent subclones for each cDNA construct in each cell line.

Long Term Trophic Signals Induced in Trk Chimeras—Trophic signals via Trk chimeras expressed in HEK293 cells were probed in survival assays using the quantitative 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide method (Table I). Cells cultured in SFM undergo apoptotic death, which can be rescued by NTFs if the cells express functional NTF receptors.

Control HEK293 cells transfected with wild type TrkB exhibited the expected survival profile (Table I, rows 7–9), where NGF protected TrkA-expressing cells, BDNF protected TrkB-expressing cells, and NT-3 protected TrkC-expressing and TrkB-expressing cells and significantly protected TrkA-expressing cells but to a lower degree. Untransfected HEK293 cells did not survive in SFM supplemented with NTFs (Table I, row 1).

HEK293 cells expressing the 1.1 chimera did not survive with NGF but did survive with NT-3 and with BDNF (Table I, row 2). Similar data was obtained with the 2.1 chimera, except that more limited survival was seen with NT-3 (Table I, row 3). Hence, the 3’ Cys-1, LRM, and Cys-2 subdomains from TrkA or TrkB do not play a role in NGF-TrkA activation (no gain of function), nor do they play a major role in BDNF-TrkB activation (no major loss of function); but perhaps they do for NT-3-TrkB activation.

Cells expressing the 2.2 chimera had low but significant survival with NGF, NT-3, and BDNF (Table I, row 4). The lower survival induced by NT-3 and BDNF in the 2.2 chimera (compared with the 2.1 chimera) is probably due to the replace-
Receptor Subdomain Utilization by Neurotrophins

TABLE I

| Stable HEK 293 subclones | SFM | SFM + 2 nM NTF | BDNF | NT-3 |
|--------------------------|-----|----------------|------|------|
| 1. Untransfected         | 4 ± 2 | 1 ± 5          | 3 ± 2 |      |
| 2. 1.1 chimera           | 7 ± 3 | 38 ± 4a        | 41 ± 12a |     |
| 3. 2.1 chimera           | 7 ± 2 | 29 ± 5a        | 43 ± 5a |     |
| 4. 2.2 chimera           | 10 ± 1a | 11 ± 2a       | 16 ± 2a |     |
| 5. 3.1 chimera           | 42 ± 7 | 42 ± 3        | 44 ± 5 |     |
| 6. 4.1 chimera           | 42 ± 3a | 15 ± 4a       | 9 ± 7  |     |
| 7. Wild type TrkA        | 40 ± 3a | 46 ± 3a      | 57 ± 6a |     |
| 8. Wild type TrkB        | 4 ± 3 | 46 ± 3a        | 57 ± 6a |     |
| 9. Wild type TrkC        | 9 ± 3 | 53 ± 3a        | 4 ± 2  |     |

a Significant with respect to corresponding cell in SFM without neurotrophin (p < 0.02).

b There is high survival for 3.1 chimeras in SFM (39 ± 3% relative to serum); hence, NTFs do not significantly enhance survival.

ment of the IgG-C1 subdomain with that of TrkA (the only difference between the 2.2 and the 2.1 chimeras). These data suggest an activating role for the IgG-C1 subdomain of TrkB bound by NT-3 or BDNF and also suggest an activating role for the IgG-C1 subdomain of TrkA bound by NGF.

HEK293 cells expressing the 3.1 chimera are constitutively activated, and cells have significant survival in SFM (~40% compared with serum) in the absence of NTFs. Survival was not enhanced significantly in response to any NTF. These data also point to a critical role of the IgG-C1 subdomain of TrkB and suggest that this subdomain is functionally different in TrkA.

HEK293 cells expressing the 4.1 chimera had significant survival with NGF, significant but low survival with NT-3, and no survival with BDNF (Table I, row 6). Compared with wild type TrkA, NGF activates in full via the TrkA IgG-C2 subdomain, and BDNF activation absolutely requires the TrkB IgG-C2 subdomain. NT-3 may activate partially via TrkA IgG-C2 (or the remaining subdomains of TrkB) in the 4.1 chimera, but it seems to be more dependent on other subdomains.

NGF Binding to Trk Chimeras—In order to test whether there are chimeras that did not signal but bound NGF, we performed 125I-NGF binding studies (Fig. 2). We tested NGF binding with the 1.1, 3.1, and 4.1 chimeras and binding to wild type HEK293 cells as control.

Only the 4.1 chimera had specific 125I-NGF binding. The 4.1 chimera subclone 6 (4.1.6) expresses ~50,000 NGF receptors/cell with an apparent single affinity of 80 ± 24 pm (n = 3 independent assays). These binding sites were fully competed by a 250-fold excess of unlabeled NGF (Fig. 2).

The 1.1 chimera, the 3.1 chimera, and the wild type HEK293 cells showed no specific NGF binding sites (n = 3 independent assays) (data not shown). The binding assays would not have detected binding affinities significantly lower than 10 nM (which is approximately the Kd of p75). Therefore, we cannot rule out very low affinity NGF binding sites in 1.1 and 3.1 chimeras. Likewise, 125I-NGF binding assays using cells co-expressing chimeras and p75 are fruitless, because it is impossible to discriminate whether the chimera (as well as p75) binds NGF with low affinity.

Direct binding studies of 125I-NT-3 and 125I-BDNF to 4.1.6 cells could not be carried out, because these ligands were unavailable to us in radiolabeled form.

Tyrosine Phosphorylation of Chimeric Receptors in HEK293

**Cells**—Trk Tyr(P) was assayed in HEK293 transfectants after 12 min of exposure to NTFs (Fig. 3, A–E). The 1.1 and 2.1 chimeras respond well to NT-3 and BDNF but not to NGF (Fig. 3, A and B); the 2.2 chimeras respond to NGF, NT-3, and BDNF but very poorly, and the gel had to be overexposed to see the response (Fig. 3C). The 3.1 chimeras are constitutively tyrosine-phosphorylated (Fig. 3D, untreated), and there is no significant increase in response to all ligands (Fig. 3D). The 4.1 chimeras respond very well to NGF and poorly to NT-3, and they do not respond to BDNF (Fig. 3E).

Gain of NGF function and loss of BDNF function defines the IgG-C2 as the main activation subdomain, with smaller contributions from other subdomains. Interpretation for NT-3 survival is less clear, because this growth factor binds both wild type TrkA and TrkB, and we cannot discriminate which receptor is bound by NT-3 in our chimeras.

**Altered Function of Trk Chimeras in HEK293 Cells Co-expressing p75**—To assess how co-expression of p75 receptors may affect the activation of Trk chimeras, HEK293 transfectants were infected with adenoviral constructs expressing full-length p75. After 16 h, there was homogeneous expression of p75 receptors in >90% of the cells, with no death (data not shown). At 16 h postinfection, the Tyr(P) of the chimeric receptors was measured (Fig. 3, F–J).

In the presence of p75 the 1.1 chimera is activated by NGF, NT-3, and BDNF (Fig. 3P). The major difference is that in the absence of p75 NGF does not activate the 1.1 chimera (Fig. 3A).

In the presence of p75, the 2.1 chimera is activated well by NT-3 and poorly by BDNF and NGF (Fig. 3C). The major difference is that in the absence of p75 (Fig. 3B) BDNF can activate the 2.1 chimera better.

In the presence of p75, the 2.2 chimera does not respond to any NTF (Fig. 3H). There are no major differences with data in the absence of p75 (Fig. 3C). In both cases, the gels had to be overexposed to see any limited response to ligand.

In the presence of p75, the 3.1 chimera is still constitutively activated (Fig. 3I, Untre.) but to a lower degree than when p75 is absent (Fig. 3D, Untre.).
In the presence of p75, the 4.1 chimeras respond well to NGF and NT-3 and do not respond to BDNF (Fig. 3). The major difference is that NT-3 activation (relative to NGF) is higher in the presence of p75 than in the absence of p75 (Fig. 3E).

In all chimeras, ligand-independent Tyr(P) of the receptors (even the constitutively activated 3.1 chimera) was substantially lower after expression of p75 (see untreated (Untre.) lanes for all panels). These data suggest that unbound p75 can inhibit constitutive Trk activation and that p75 can alter the ligand-dependent activation subdomains of Trk receptors.

**Trophic Function of Trk Chimeras in a Neuronal Cell Line**

**Fig. 3.** Ligand-induced tyrosine phosphorylation of chimeric receptors. HEK293 cells expressing the indicated chimeric Trk receptor alone (A–E) or with co-expression of p75 (F–J) were untreated or were treated with a 2 nM concentration of the indicated NTFs for 12 min. Cell lysates were fractionated in 7.5% SDS-polyacrylamide gel electrophoresis and Western blotted with α-Tyr(P) mAb 4G10.

**Table II.** Ligand-mediated survival of nnr5 cells expressing Trk receptors

| Stable nnr5 subclones<sup>a</sup> | SFM + 2 nM NTF |
|------------------------------|---------------|
| NGF                         | NT-3          | BDNF          |
| 1. Untransfected            | 3 ± 6         | 3 ± 7         | 7 ± 6         |
| 2. 1.1 chimera              | 20 ± 2<sup>b</sup> | 10 ± 4      | 10 ± 3        |
| 2. 2.1 chimera              | 12 ± 3        | 14 ± 3<sup>b</sup> | 15 ± 8     |
| 4. 2.2 chimera              | 9 ± 4         | 11 ± 7        | 10 ± 4        |
| 5. 4.1 chimera              | 14 ± 2<sup>b</sup> | 20 ± 3<sup>b</sup> | 9 ± 6      |
| 6. Wild type TrkA           | 37 ± 2<sup>b</sup> | 21 ± 2<sup>a</sup> | 6 ± 1        |
| 7. Wild type TrkB           | 5 ± 1         | 11 ± 1<sup>b</sup> | 26 ± 3<sup>b</sup> |

<sup>a</sup> Stable 3.1 chimera-expressing clones were not obtained; transfectants seem to differentiate and stop growing.

<sup>b</sup> Significant with respect to corresponding SFM (p < 0.02).

The IgG-C1 Subdomain Is Surface-exposed in the Receptor Extracellular Domain—Given the intriguing results seen for TrkA IgG-C1, we predicted that this subdomain may be surface-exposed in the tertiary structure of the receptor. This hypothesis is based on the fact that surface structures generally correlate with access to a ligand or with the ability to induce conformational changes (27, 28). Immunogenicity is an accepted criterion for testing surface exposure of receptors (29, 30).

We used anti-TrkA antiserum RTA generated by immunization with whole TrkA-ECD (25). The serum was affinity-purified using native TrkA-ECD to trap RTA immunoglobulins. These antibodies react only with surface-exposed epitopes of the extracellular domain, because the immunoglobulins directed to buried or masked epitopes would not have been affinity-purified.

HEK293 cells expressing the Trk chimeras were tested by Western blotting with purified RTA immunoglobulins (Fig. 4).

**DISCUSSION**

Our studies define four issues of neurotrophin receptor biology. First, we show that the IgG-C1 subdomain is surface-exposed and plays a regulatory role in receptor activation and perhaps in low affinity ligand binding. Second, NGF binds to the IgG-C2 domain of TrkA. Third, we identify extracellular subdomains relevant for TrkA or TrkB activation by cognate (NGF or BDNF, respectively) and heterologous (NT-3) ligands. Fourth, we show that p75 modulates the Trk subdomains necessary for ligand-dependent activation.
Nitrophenyl acetylcholine (31). The presence of p75 reportedly makes NT-3 inefficient at activating the 3.1 chimera because of the IgG-C1 domain in neurotrophin binding (7, 9). Together with the literature, our data are consistent with the literature, where the interactions of NGF—p75 enhance wild type TrkA ligand selectivity and affinity (3, 32, 33) and affect TrkA biological activity (20–22, 24).

Additionally, we cannot exclude a conformational effect of p75 upon the neurotrophins themselves. This has been proposed previously, wherein an anti-NGF antibody blocked all of the neuritogenic but not the trophic action of NGF (20–22, 24).

Regulation of Activity by the IgG-C1 Subdomain—It is generally accepted that receptor dimerization and conformational changes must occur for activation and signaling. Regulatory subdomains have been shown to inhibit ligand-independent dimerization activation of other receptors (34, 35), and the steric constraints are removed by conformational changes upon ligand binding.

The IgG-C1 subdomain of TrkA plays a key role in regulation of ligand-independent activation. It is also attractive to speculate that the p75-mediated reduction of ligand-independent activation seen in all Trks may be due to an interaction of p75 with the TrkA Cys-1-rich motif. Our data are consistent with the literature, where the interactions of NGF—p75 enhance wild type TrkA ligand selectivity and affinity (3, 32, 33) and affect TrkA biological activity (20–22, 24).

The Role of p75 in BDNF and NT-3 Activation—The subdomains used by BDNF and NT-3 to activate TrkB change when p75 is co-expressed. In the presence of p75 co-receptors, the N-terminal subdomains of TrkB (LRM and Cys-2) are necessary for optimal activation by BDNF (Fig. 3, compare B with C). Also, whether or not p75 co-receptors are present, NT-3 activates the chimeric 4.1 receptor (IgG-C2 subdomain of TrkA) to a level comparable with wild type TrkA but different from wild type TrkB.

The presence of p75 reportedly makes NT-3 inefficient at activating wild type TrkA (18). Therefore, the role of p75 in enhancing NT-3 activation of the 4.1 chimera could be due either to enhanced NT3-TrkA IgG-C2 interactions or to NT-3 binding to other subdomains of TrkB (31).

Changes induced by p75 in TrkB subdomain ligand docking sites explain the mechanism wherein BDNF and NT-3 activate wild type TrkB equally, but in the presence of p75 co-receptors BDNF activates better than NT-3 (19).

TrkB Activation Subdomains of BDNF and NT-3—Based on decrease or loss of BDNF and NT-3 responsiveness in the 4.1 chimera (compared with wild type receptors), we conclude that BDNF-TrkB and NT-3-TrkB activation is mediated by the IgG-C2 subdomain. Based on a decrease in BDNF and NT-3 responsiveness in the 2.2 chimera compared with the 2.1 chimera, we conclude that BDNF-TrkB and NT-3-TrkB activation also requires the IgG-C1 subdomain. The functional role of the IgG-C1 subdomain is further supported by ligand-independent activation of the 3.1 chimera.

In related studies of TrkB/TrkC chimeras, only the IgG-C2 subdomain of TrkB bound BDNF, and the IgG-C1 did not (7). If the IgG-C1 affects activity in the absence of direct ligand binding, this would suggest a regulatory or allosteric role in maintaining receptor conformation, and this possibility is supported by a surface exposure of IgG-C1 within the Trk architecture.

Conclusions and Putative Model—We conclude that (i) Trk receptors have distinct ligand binding and regulatory subdomains, (ii) p75-mediated regulation of activation requires the N-terminal subdomains of Trk, (iii) there are multiple NTF

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**TABLE III**

| Table: Summary of survival and Tyr(P) data |

| Trk A/B Constructs | p75 co-expression | Ligand-induced activation of chimeric receptors* |
|---------------------|-------------------|-----------------------------------------------|
|                     |                   | BDNF | NT-3 | NGF |
| 1.1 Cys-1           | No                | Low  | High | High |
| 2.1 LRM-Cys-2       | Yes               | High | Medium | Low |
| 2.2 LRM-Cys-2-IgG-C1| No                | Low  | High | Low |
| 3.1 IgG-C1*         | Yes               | Low  | Nil  | Nil |
| 4.1 IgG-C2          | No                | Medium | High | Nil |

*a High > medium > low > nil. Summarized from p Tyr(P) and cell survival assays. These two assays are generally consistent but not necessarily linear, and kinetics may vary (24, 26). Italics and boldface type show very significant changes. Underlining shows significant changes.

*b Activation above that which is detected independent of ligand.
docking sites for Trk (some are preferred and some allosteric (perhaps corresponding to high and low affinity, respectively)), and (iv) regulation of activation by the TrkA IgG-C1 subdomain is different from regulation by the TrkB IgG-C1 subdomain, based on the 3.1 chimera effect.

We postulate a possible model of two binding sites on Trk for NTFs, modulated by the co-receptor p75 (Fig. 5). In this model, receptor stoichiometry and possible allosteric changes are simplified or not shown. NGF binding to the IgG-C2 domain causes a conformational change in wild type TrkA (Fig. 5B). The conformational effect on the architecture may occur at the surface-exposed IgG-C1 subdomain, which could “rotate”, as described for other receptors (34, 35, 39).

Co-expression of p75 affords high affinity binding via a second site on the N-terminal subdomain of TrkA (Fig. 5C). Whether this is due to altered conformation of NGF (induced by p75-NGF interactions) or TrkA (induced by p75-TrkA interactions) remains to be determined, and the literature is inconclusive. Further, NGF could bind the second receptor site on the same receptor or on a juxtaposed receptor.

Consistent with the putative model, the 1.1 chimera co-expressing p75 can be activated by NGF (Fig. 5D), although this chimera does not bind NGF (or it binds with affinity lower than nanomolar). Also consistent with the model, the 3.1 chimera is constitutively activated (Fig. 5E), possibly by inducing a “rotation” analogous to ligand binding. Last, the 4.1 chimera binds NGF with intermediate or high affinity and is activated fully by NGF (Fig. 5F).

The Trk subdomains identified in this study may be receptor functional “hot spots,” a concept proposed for other receptors (34, 35, 39). Trk receptor signaling, the identification of multiple binding subdomains for the neurotrophins, and the regulation of subdomain utilization by receptors and ligands raises the possibility of a complex, multistage process whose study will give a better understanding of neurotrophin structure-activity relationships and signal transduction.

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REFERENCES

1. Eide, F. F., Lowenstein, D. H., and Reichardt, L. F. (1993) Exp. Neuro 121, 200–214
2. Barbacid, M. (1994) J. Neurobiol. 25, 1386–1403
3. Jing, S., Tapley, P., and Barbacid, M. (1992) Neuron 9, 1067–1079
4. Kaplan, D. R., and Stephens, R. M. (1994) J. Neurobiol. 25, 1404–1417
5. Schneider, R., and Schweiger, M. (1991) Oncogene 6, 1807–1811
6. Perez, P., Coll, P. M., Hempstead, B. L., Martin-Zanca, D., and Chao, M. (1995) Mol. Cell. Neurosci. 6, 97–105
7. Urfer, R., Tsoulias, P., O’Connell, L., Shelton, D., Parada, L., and Presta, L. (1995) EMBO J. 14, 2795–2805
8. MacDonald, J. L. S., and Meakin, S. O. (1996) Mol. Cell. Neurosci. 7, 371–390
9. Urfer, R., Tsoulias, P., O’Connell, L., Hongo, J., Zhao, W., and Presta, L. (1998) J. Biol. Chem. 273, 5829–5840
10. Holden, P. H., Ao, V., Robertson, A. G. S., Clarke, A. R., Tyler, S., Bennett, G. S., Brian, S. D., Wilcock, G. K., Allen, S. J., Smith, S. K. F., and Dawbarn, D. (1997) Nat. Biotechnol. 15, 668–672
11. Windisch, J. M., Marksteiner, R., Lang, M. E., Auer, B., and Schneider, R. (1995) Biochemistry 34, 11256–11263
12. Windisch, J. M., Marksteiner, R., and Schneider, R. (1995) J. Biol. Chem. 270, 28133–28136
13. Kadari, A., Windisch, J., Ebendal, T., Schneider, R., and Humpel, C. (1997) J. Neurosci. Res. 50, 402–412
14. Heldin, C. H. (1995) Cell 80, 213–223
15. Woo, S., Whalen, C., and Neet, K. (1998) Protein Sci. 7, 1006–1016
16. Wiesmann, C., Utsch, M. H., Bass, S. H., and Vos, A. M. D. (1999) Nature 401, 184–188
17. Bomji, S. X., Majdan, M., Ponziai, C. D., Belliveau, D. J., Aloyz, R., Kohl, J., C. G. Causin, and Miller, F. D. (1996) J. Cell Biol. 134, 911–923
18. Brennan, C., Rivas-Plata, K., and Landis, S. (1999) Nat. Neurosci. 2, 699–705
19. Bibel, M., Hoppe, E., and Barde, Y. (1999) EMBO J. 18, 616–623
20. MacLewie, J. L., and Barker, P. A. (1997) J. Biol. Chem. 272, 23547–23551
21. Maluarte, H., and Saragovi, H. U. (1997) J. Neurosci. 17, 6031–6037
22. Kohn, J., Aloyz, R., Toma, J., Haak-Frendscho, M., and Miller, F. (1999) J. Neurosci. 19, 5395–5408
23. Mufson, E. J., Lavine, N., Jaffar, S., Kordower, J. H., Quirion, R., and Saragovi, H. U. (1997) Exp. Neuro 146, 91–103
24. Saragovi, H. U., Zheng, W. H., Maluarte, H., DiGugliemo, G. M., Mawal, Y. R., Kamen, A., Woo, S. H., Cuello, A. C., Debeer, T., and Neet, K. (1998) J. Biol. Chem. 273, 34933–34940
25. Clary, D. O., Weskamp, G., Austin, L. R., and Reichardt, L. F. (1994) Mol. Biol. Cell 5, 549–563
26. Cunningham, M. E., Stephens, R. M., and Greene, L. A. (1997) *J. Biol. Chem.* 272, 10957–10962
27. Saragovi, H. U., Greene, M. I., Chrusciel, R. A., and Kahn, M. (1992) *Bio/Technology* 10, 773–778
28. Saragovi, H. U., and Burgess, K. (1999) *Expert Opin. Ther. Patents* 9, 737–751
29. Morgan, D. O., and Roth, R. A. (1986) *Biochemistry* 25, 1364–1371
30. Soos, M., Siddie, K., Baron, M., Heward, J., Luzio, J., Bellatin, J., and Lennox, E. (1986) *Biochem. J.* 235, 199–208
31. Ninkina, N., Grashchuck, M., Buchman, V., and Davies, A. (1997) *J. Biol. Chem.* 272, 13019–13025
32. Barker, P. A., and Shooter, E. M. (1994) *Neuron* 13, 203–215
33. Mahadeo, D., Kaplan, D., Chao, M., and Hempstead, B. (1994) *J. Biol. Chem.* 269, 6884–6991
34. Livnah, O., Johnson, D. L., Stura, E. A., Farrell, F. X., Barbone, F. P., You, Y., Liu, K. D., Goldsmith, M. A., He, W., Krause, C. D., Pestka, S., Jelliffe, L. K., and Wilson, I. A. (1998) *Nat. Struct. Biol.* 5, 993–1004
35. Remy, I., Wilson, I., and Michnick, S. (1999) *Science* 283, 990–993
36. Arevalo, J., Conde, B., Hempstead, B., Chao, M., Martin-Zanca, D., and Perez, P. (2000) *Mol. Cell. Biol.* 20, 5968–5916
37. Arevalo, J., Conde, B., Hempstead, B., Chao, M., Martin-Zanca, D., and Perez, P. (2001) *Oncogene* 20, 1229–1234
38. Coulter, F., Kumar, R., Ernst, M., Klein, R., Martin-Zanca, D., and Barbacid, M. (1990) *Mol. Cell. Biol.* 10, 4202–4210
39. Wells, J. A. (1996) *Science* 273, 449–450