Neurotrophins are important for the development and maintenance of the vertebrate nervous system, mediating their signal into the cell by specific interaction with tyrosine kinase receptors of the Trk family. The extracellular portion of the Trk receptors has been previously proposed to consist of a cysteine-rich motif, a leucine-rich motif, a second cysteine-rich motif followed by two immunoglobulin-like domains. Earlier studies have shown that a major neurotrophin-binding site in the Trk receptors resides in the second immunoglobulin-like domain. Although the individual amino acids in TrkA involved in binding to nerve growth factor (NGF) and those in TrkB involved in binding to neurotrophin-3 have been mapped in this domain, the Trk amino acids that provide specificity remained unclear. In this study, a minimum set of residues in the human TrkC second immunoglobulin-like domain, which does not bind nerve growth factor (NGF), were substituted with those from human TrkA. The resulting Trk variant recruited binding of NGF equivalent to TrkA, maintained neurotrophin-3 binding equivalent to TrkC, and also bound brain-derived neurotrophin, although with lower affinity compared with TrkB. This implies that the amino acids in the second immunoglobulin-like domain that determine Trk specificity are distinct for each Trk.

The neurotrophins form a highly homologous family of growth factors responsible for differentiation, survival, and function of neurons sensitive to their presence (reviewed in Refs. 1–5). These molecules may also play a role outside the nervous system (6, 7). The mammalian members of this family include nerve growth factor (NGF),1 brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4/5 (NT4/5) (reviewed in Ref. 8). Neurotrophins bind to two classes of receptors, tyrosine kinases encoded by the neurotrophin-3 (NT4/5) (reviewed in Ref. 8). Neurotrophins bind to two classes of receptors, tyrosine kinases encoded by the Trk gene family and the Trk gene family and p75NTR (1–4, 9). Neurotrophin binding induces autophosphorylation of the Trk receptors that triggers the subsequent steps in the signal transduction cascade (reviewed in Ref. 4). Each Trk receptor can discriminate between the different neurotrophins as follows: TrkC interacts with NT-3 (10), TrkB interacts primarily with BDNF and NT-4/5 (11, 12), and TrkA interacts primarily with NGF (13), although TrkB and TrkA can bind NT-3 (12, 14).

The domain organization of the extracellular portion of the Trk receptors has been proposed based on sequence information (15). According to this proposal, the extracellular portion of the Trk receptors is comprised of a cysteine cluster, a leucine-rich motif, a second cysteine cluster followed by two immunoglobulin-like domains. Previous studies have shown that the second immunoglobulin-like domain of the Trk receptors is involved in binding to their respective neurotrophins. Deletion of the second immunoglobulin-like domain in TrkA (16, 17) and TrkC (16) abrogates neurotrophin binding. Exchanging the second immunoglobulin-like domain from TrkA or TrkB into TrkC resulted in high-affinity NGF and BDNF binding, respectively (16), and exchanging the two immunoglobulin-like domains from TrkA into TrkB also transferred NGF binding (18). In cells transfected with receptors comprising only the two immunoglobulin-like domains of TrkA (17) or TrkC (16) or only the second immunoglobulin-like domain of TrkC (16), these truncated receptors bound neurotrophin and exhibited autophosphorylation. In addition, a fragment of TrkA comprising the two immunoglobulin-like domains has been shown to bind NGF and inhibit neurite outgrowth (19, 20).

A second neurotrophin-binding site on Trk receptors, the leucine-rich motif (LRM) domain, has also been implicated in binding neurotrophins (21–25). Peptides corresponding to segments of the LRM domain can bind to neurotrophins and inhibit neurotrophin binding to Trk receptors, although only within a Kᵸ value in the micromolar range (24). In contrast to the studies showing that transferring the second immunoglobulin-like domain among Trk receptors also transfers neurotrophin binding specificity (16, 18), no BDNF binding was observed when the TrkB LRM domain was substituted into TrkC (16) nor was NGF binding recruited when the TrkA LRM domain was substituted into TrkC (26) or TrkB (18). Although the role of the LRM domain in binding neurotrophins remains unclear, the function of this domain was suggested in a study in which cells were transfected with TrkA receptors in which the LRM domain was deleted. These cells bound NGF, showed autophosphorylation of the Trk receptor, and activation of the Shc-dependent Ras pathway, but they failed to fasciculate and showed delayed aborization (17).

The amino acids in the second immunoglobulin-like domain of TrkA involved in binding NGF and those in TrkC involved in binding NT-3 have been previously mapped (26), although the amino acids that controlled specificity were not elucidated. In order to determine the residues that control specificity of TrkA for NGF, a TrkC-based variant was generated by replacing TrkC residues with the minimum set of TrkA residues necessary to recruit NGF binding equivalent to native TrkA. Unexpectedly, this TrkC-based variant maintained NT-3 binding equivalent to native TrkC and also bound BDNF, although less well than native TrkB. This implies that the amino acids in the...
Trk receptors that determine specificity for their respective neurotrophins occupy distinct, separate positions in the second immunoglobulin-like domain sequence.

**EXPERIMENTAL PROCEDURES**

**Plasmid and Protein Preparation—**Plasmids encoding the native human TrkA-immunoadhesin and native human TrkC-immunoadhesin were used as the template for site-directed mutagenesis to generate plasmidic coding for the variants, as described previously (26). Plasmids were transfected into human embryonic kidney 293 cells, and Trk proteins were expressed, purified, and quantified as described previously (26).

**Binding of NT-3 to TrkC Variants—**The binding of TrkC variants to NT3 was evaluated using an enzyme-linked immunosorbent assay method analogous to one previously described (26). Microwell plates (NUNC, Denmark) were coated overnight at 4 °C with 100 μl per well of a 5 μg/ml solution of goat F(ab’)2 anti-human IgG (Fc) (Cappel-ICN Immunobiologics, Costa Mesa, CA) in 50 mM carbonate buffer, pH 9.6. The plates were then washed with wash buffer (phosphate-buffered saline (PBS), 0.05% Tween 20), and excess binding sites were blocked with 200 μl/well of PBS containing 0.5% bovine serum albumin + 0.05% Tween 20 (PBS/BSA/T) for 1–2 h at ambient temperature. Stock solutions of NT3 wild type or variant immunoadhesins in PBS/BST/T (1 or 10 μg/ml, as noted) were serially diluted with PBS/BST/100 μl/well was added to the appropriate wells on the plate and incubated for 1 h at ambient temperature. The plates were then washed, and 100 μl/well of a 100 ng/ml solution of biotinylated human NT-3 (Genentech, Inc.) in PBS/BST/T was added and incubated at ambient temperature for 1 h. Binding of the conjugate was detected with 5 ng of o-phenylenediamine tablets (Sigma) dissolved in PBS containing 4 mM H2O2 (100 μl/ml) solution) (Genentech, Inc.) and a 490 nm filter for absorbance and 405 nm reference filter.

**Binding of NGF to TrkC Variants—**The binding of TrkC variants to NGF was evaluated using an immunosorbent assay analogous to the method described above. Plates were coated overnight at 4 °C with human NGF (100 μl/well of a 1 μg/ml solution) (Genentech, Inc.), washed, and excess binding sites blocked as described above. Serial dilutions of wild type TrkC (4 μg/ml) and 50 μl of variant solutions (64 μg/ml to 3.9 ng/ml) were read with an automated plate reader (Wallac, Turku, Finland) in 50 μl/well of a 100 ng/ml solution of goat F(ab’)2 anti-human IgG (Fc) (Cappel-ICN Immunobiologics), and o-phenylenediamine substrate as described above.

**Construction of Stable, Transfected NIH3T3 Cells—**The extracellular domains for variants C13 and C1R1 were amplified using polymerase chain reaction, fused to DNA coding for the TrkA transmembrane and cytosolic domains, and subcloned into the mammalian expression vector, pMEXPneo (27), using SalI and ClaI sites. Subconfluent NIH3T3 cells were transfected with the plasmids using the Lipofectin (Roche Molecular Biochemicals) according to manufacturer's instructions. At least 24 single colonies for each of the constructs were selected for G418 resistance, expanded, and assayed for receptor expression by immunoprecipitation with pan-Trk antibody 45 (a gift of Dr. Barbara Hempstead) as described previously (28).

**Autophosphorylation of Trk Variants on NIH3T3 Cell Lines—**Approximately 1 × 10⁷ cells were treated at 37 °C for 5 min with the appropriate neurotrophin at concentrations indicated in the figures. The cells were then lysed with 1% Nonidet P-40 (Sigma) lysis buffer, immunoprecipitated with pan-Trk 45 or with Trk C-14 (Santa Cruz Biotech, West Grove, PA) using manufacturer's instructions. At least 24 colonies for each of the constructs were selected for G418 resistance, expanded, and assayed for receptor expression by immunoprecipitation with pan-Trk antibody 45 (a gift of Dr. Barbara Hempstead) as described previously (28).

**Fluorescence Microscopy of Tagged Receptors—**Infected PC12 cells were fixed for 10 min in 4% paraformaldehyde. Cells were then washed in PBS and incubated for 1 h with a 1:300 dilution of stock anti-EGFP antibody (Quantum, Montréal, Canada). Secondary antibody used for staining was 1:200 dilution of stock Cy3-conjugated donkey anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA). Images were acquired using a CCD camera (Optronix Engineering, Goleta, CA) coupled to a PowerMacintosh (Apple) with a CG-7 frame grabber (Scion, Frederick, MD) running Image Pro-Plus software (Media Cybernetics, Silver Spring, MD). Single images were assembled with Adobe Photoshop/Illustrator.

**RESULTS**

A previously described TrkA/TrkC chimera, SSA (16), was used as the initial template to generate the variants in this study; SSA has TrkC domain 5 (second immunoglobulin-like domain) exchanged with that from TrkA (i.e., domains 1–4 from TrkA and domain 5 from TrkC). SSA bound NGF approximately 6-fold reduced compared with full-length TrkC (EC50 SSA/EC50 TrkA = 6.22 ± 1.7, n = 8). This contrasts to equivalent binding of SSA and TrkA found in a previous study (16), however, the studies differ in the assay used to measure NGF binding.

Initially, groups of residues in SSA domain 5 that differed from human TrkC were exchanged for their TrkC counterparts. For each group exchanged, the new variant was evaluated for NGF binding. Exchanging amino acids at positions 305, 306, 308, 312, 313, 328, 330, 332, 337, 338, and 365 (residue numbers in the text refer to human TrkC as noted in Fig. 1) did not affect NGF binding (data not shown). In addition, when all of these positions were simultaneously changed to the TrkC sequence (variant C13), NGF binding remained equivalent to wild type TrkC (Table I and Fig. 2A). Variant C13 functioned as equivalent to C13; individually changing these residues to their human TrkC counterparts reduced NGF binding by 2–4-fold (Table 1). Other residues that affected binding when changed to their TrkC counterparts were Prp22, Ser254, Asp326, His356, Met356, Asp397, Phe402, Glu405, and Asp406 (Table 1). Note that no individual changes were made in the segment connecting β-strands C and E (TrkC residues 340–360). The crystal structures of domain 5 of TrkA and TrkC (30) show that this segment differs between TrkC and TcrA not only in the nature of the amino acids but also in its size and conformation; therefore, changing residues in this segment in a one-to-one correspondence was not possible.

Based on the results in Table 1, all TrkA residues that did not have an effect on binding were simultaneously changed from...
Fig. 1. Sequence alignment of domain 5 (second immunoglobulin-like domain) of human TrkA, human TrkC, and TrkC-based variants. Residue numbers for TrkA and TrkC precede each sequence and dots mark each 10. β-Strands in domain 5 are overlined and labeled A–G; β-strands were determined from the crystal structure of human TrkC domain 5 (30). For TrkC-based variants, differences from TrkC are shaded. For variant C13, residues that were determined to be important for binding NGF (Table I) are noted with a +; those that were not are noted with a − below the C13 sequence.

![Sequence Alignment](https://example.com/sequence_alignment.png)

**TABLE I**

| Variant | EC50 C13 EC50 variant | TrkA |
|---------|----------------------|------|
|         | Mean S.D. n           |      |
| TrkA    |                      |      |
| His311  | (291) → Glu          | 1.02 0.28 24 |
| Val314  | (294) → Glu          | 0.93 0.02 2 12 |
| Glu315  | (295) → Leu          | 0.25 0.01 2 14 |
| Met316  | (296) → Arg          | 0.55 0.11 2 3 |
| His317  | (297) → Leu          | 0.25 0.02 2 7 |
| His318  | (298) → Glu          | 0.27 0.01 2 15 |
| Pro320  | (302) → Glu          | 0.37 0.02 2 1 |
| Ser324  | (304) → Val          | 0.19 0.06 4 >100 |
| Asp326  | (306) → Arg          | 0.42 0.02 2 11 |
| Arg324  | (314) → His          | 0.48 0.02 2 5 |
| His325  | (315) → Gly          | 0.89 0.04 2 3 |
| Glu392  | (350) → Lys          | 0.57 0.02 2 27 |
| Val347  | (354) → Tyr          | 1.39 0.14 2 6 |
| Leu379  | (362) → Ile          | 0.83 0.02 2 24 |
| Ala391  | (372) → Glu          | 0.72 0.04 2 2 |
| Thr392  | (373) → Thr          | 0.81 0.07 2 NE |
| Gly393  | (374) → Thr          | 1.04 0.17 2 2 |
| Ser398  | (375) → Asn          | 1.19 0.08 2 2 |
| Ala399  | (376) → Asn          | 1.18 0.10 2 NE |
| Ser399  | (377) → Thr          | 0.86 0.09 2 48 |
| Ala393  | (378) → Gly          | 0.90 0.07 4 48 |
| Ala394  | (379) → Asn          | 0.84 0.14 4 NE |
| Met396  | (382) → Leu          | 1.73 0.35 4 1 |
| Thr397  | (383) → Lys          | 0.29 0.02 4 NE |
| Asp396  | (384) → Arg          | 0.58 0.24 4 1 |
| Asn398  | (385) → Glu          | 0.82 0.20 4 1 |
| Glu401  | (386) → Pro          | 1.18 0.23 4 5 |
| Phe402  | (387) → Val          | 0.32 0.11 4 NE |
| Asn403  | (388) → Asp          | 0.90 0.08 4 5 |
| Pro404  | (389) → Glu          | 0.96 0.12 4 NE |
| Glu405  | (390) → Val          | 0.57 0.06 4 18 |
| Asp406  | (391) → Ser          | 0.62 0.14 4 18 |
| Ile408  | (391) → Thr          | 0.72 0.04 2 8 |

TrkA to TrkC sequence, except at position 371. The resulting variant CR1 bound NGF 2.5-fold better than C13 (Fig. 1 and Table I). Position 371 is part of domain 5 loop EF, and most of this loop is conserved among TrkA, TrkB, and TrkC, although position 371 varies. Since it had been previously shown that loop EF plays a major role in neurotrophin binding to TrkA and TrkC and that Val371 was a major determinant in NGF binding to TrkA (26), Val371 was retained in further variants.

Two sections that differ in length and sequence between TrkA and TrkC are segment C-E and the C-terminal half (i.e. residues 408–419) of the juxtamembrane segment connecting the end of domain 5 with the transmembrane segment (Fig. 1). When segment C-E was changed from TrkA to TrkC sequence, NGF binding was reduced 9-fold (CR2, Table II) and when the C-terminal half of the juxtamembrane segment was changed to TrkC sequence, NGF binding was reduced by almost 4-fold (CR3, Table II). The role of the amino acid at position 319 was also evaluated; when changed from TrkA Trp319 to TrkC Trp319, binding of NGF and NT-3 to wild type and variant TrkA and TrkC. A, binding of human NGF to TrkA (open square, large dashed line), TrkC (open triangle, solid line), and variant C13 (filled circle, small dashed line), and variant CR1 (filled square, small dashed line). B, binding of human NT-3 to TrkA (open square), TrkC (open triangle, solid line), and variant C13 (filled circle, large dashed line), and variant CR1 (filled square, small dashed line).
His319, NGF binding was reduced 2-fold (CR4, Table II). In the Trk domain 5, crystal structures residue 319 is buried between loop AB and the N-terminal portion of the juxtamembrane segment (Fig. 3) (30).

The variant with optimal binding, CR1, bound NGF better than native TrkA (Table II and Fig. 2A). This variant included the entire TrkA segment C-E, most of TrkA loop AB, and the entire TrkA C-terminal half of the juxtamembrane segment (residues 408–419). Additional residues required were as follows: three in β-strand B (Pro322, Ser324, and Asp326), one in loop EF (Val377), and three in the N-terminal half of the juxtamembrane segment (Met396, Asp397, and Phe402). Comparing the sequence of CR1 with that of TrkC, a total of 37 TrkC residues was replaced with TrkA sequence (33%) in domain 5, of which almost half (17) were in segment C-E.

Unexpectedly, variant CR1 maintained binding to NT-3 that was equivalent to native TrkC (Table II and Fig. 2B). Variant C13, however, showed reduced NT-3 binding compared with native TrkC (Table II and Fig. 2B). Replacing the TrkA sequence with TrkC sequence at residue 319 (CR4) or in segment C-E (CR2) did not improve NT-3 binding (Table II). In contrast, changing the juxtamembrane segment to TrkC reduced NT-3 binding (CR3).

Domain 5 from C13 and CR1 were substituted for domain 5 in native TrkA (variants C13A and CR1A; Table II). Having domains 1–4 from TrkA (instead of TrkC) did not improve binding or specificity. Hence the amino acids most important for NGF and NT-3 binding and specificity seem to reside in domain 5, in agreement with previous studies (18–20). If amino acids outside of domain 5 are important for binding and/or specificity, they must have a minimal effect.

The ability of C13 and CR1 to bind NGF and NT-3 and elicit a biological response was evaluated by fusing the extracellular domains of these variants to the transmembrane/intracellular domain of TrkA. After generation of stable, transfected NIH3T3 cells expressing the chimeric proteins, the cells were evaluated for protein expression level (Fig. 4). Three cell lines of each variant were then pulsed for 5 min with NGF or NT-3, followed by lysis, immunoprecipitation, and detection of tyrosine phosphorylation of the intracellular domain (Fig. 5). All
three C13 cell lines expressed equivalent amounts of protein (Fig. 4) and exhibited NGF-induced phosphorylation levels similar to TrkA (Fig. 5A). NT-3-induced phosphorylation of C13 was reduced compared with NGF but was more pronounced than that of TrkA (Fig. 5A). For the three CR1 cell lines, different levels of expression were found (Fig. 4). As with C13, CR1 cell lines showed tyrosine phosphorylation induced by both NGF and NT-3 and the higher expressing cell line exhibited the most phosphorylation (Fig. 5B). A cell line expressing TrkC responded to NT-3 but not NGF, as expected (Fig. 5C).

One cell line from the C13 and CR1 variants was chosen to evaluate dose dependence. The C13–9 cell line showed dose dependence for both NGF and NT-3 with NGF eliciting a stronger response at a given neurotrophin concentration (e.g. compare 10 ng/ml neurotrophin in Fig. 6A). In contrast, BDNF at 200 ng/ml did not elicit phosphorylation of C13-9 (Fig. 6A). Cell line CR1-5 also showed a dose dependence for NGF and NT-3 (Fig. 6B). However, in contrast to C13, the CR1-5 cell line also responded to BDNF, although a higher concentration of neurotrophin was required to elicit the same level of phosphorylation (Fig. 6C).

The ability of the various neurotrophins to induce neurite outgrowth in transfected PC12 cells was also evaluated (Fig. 7). Whereas PC12 cells naturally possess TrkA, it has been shown previously that in PC12 cells transfected with TrkC, NT-3 leads to a significant induction of neurites during the first 3 days after application of neurotrophin, whereas NGF does not; however, at 10 days NGF and NT-3 induce similar neurite outgrowth (32). Hence all transfected PC12 cells were evaluated for neurites after only 2 days. Neurite outgrowth in C13-PC12 and CR1-PC12, but not TrkB-PC12 and TrkC-PC12, cells could be induced by NGF (Table III); if NGF binding to native TrkA on the PC12 cells was responsible for neurite extension, then the TrkC-PC12 and TrkB-PC12 cells should have exhibited neurite induction similar to C13-PC12 and CR1-PC12. In contrast, only TrkC-PC12 and CR1-PC12 cells were induced by NT-3 (Table III), in agreement with NT-3 binding better to CR1 than to C13 (Table II). BDNF elicited neurite outgrowth in CR1-PC12 and TrkB-PC12 cells but required a 5-fold increase in BDNF concentration (100 ng/ml) to elicit a response equivalent to BDNF for TrkB-PC12 cells (20 ng/ml) (Table III).

DISCUSSION

Normally, NGF binds only to its cognate Trk receptor, TrkA (13), whereas NT-3 can bind not only to TrkC but also to TrkA and TrkB (10, 14, 31). TrkA and TrkC domain 5 have only 37% homology (44 residues out of 119), and one might expect that the differences affect the specificity of these two receptors. In order to determine the TrkA residues governing its specificity, a variant of TrkC was generated that bound NGF equivalent to (or slightly better than) TrkA. This was accomplished by exchanging TrkC residues for TrkA residues in most of loop AB, all of segment C-E, and the C-terminal half of the juxtamembrane segment. Additional exchanged residues required were as follows: three in β-strand B (Pro322, Ser324, and Asp326), one in loop EF (Val371), and three in the N-terminal half of the juxtamembrane segment (Met396, Asp397, and Phe402) (CR1, Table II and Fig. 1).

In conjunction with data that the N terminus of NGF plays an important role in TrkA binding and specificity whereas it does not for NT-3/TrkC (31, 33–35), it was previously suggested that the central β-strand residues (including the conserved Arg103) of neurotrophins interact with Trk loop EF, whereas the N-terminal residues of NGF interact with loop AB and/or β-strand B (26). Recently the crystal structures of domain 5 of
TrkA, TrkB, and TrkC have been published (30). Even more recently, the crystal structure of NGF bound to TrkA domain 5 has been reported (36). Based on these crystal structures, mapping of the residues important in TrkA and TrkC for binding their respective ligands (26), and on the set of residues transferred in this study, several areas in TrkA domain 5 seem to be important for binding and specificity as follows: loop AB, β-strand B, segment C-E, loop EF, and the juxtamembrane segment.

In loop AB and β-strand B all exposed residues were required to recruit NGF binding in the TrkC variant (Table I and Figs. 1 and 3). In the NGF/TrkA domain 5 crystal structure (36), the NGF N terminus indeed interacts with loop AB and β-strand B. However, in β-strand B only Pro322 makes direct contact with NGF although Ser324 and Asp326 were also required for NGF binding (Table I). In the NGF/TrkA (36) and TrkA (30) crystal structures, the side chains of Ser324 and Asp326 form intramolecular hydrogen bonds to side chains in segment C-E suggesting...

**TABLE III**

Induction of neurite extension on PC12 cells transfected with EGFP-fused receptors

Retrovirus containing the different receptors fused to EGFP were used to infect PC12 cells. One day post-transfection, neurotrophins were added to the medium at the indicated concentration. Two days later the cells were fixed and counted. 150 cells from each experiment were counted; each neurotrophin/receptor combination was done in triplicate, except for TrkB done in duplicate. Untransfected PC12 cells did not show any neurite extension after 2 days when exposed to NGF, NT-3, or BDNF.

| Variant       | NGF 20 ng/ml | NT-3 20 ng/ml | BDNF 20 ng/ml | Neurotrophin 0 ng/ml |
|---------------|--------------|---------------|---------------|---------------------|
|               | Mean S.D. n  | Mean S.D. n   | Mean S.D. n   | Mean S.D. n         |
| TrkC-EGFP     | 4 1 3        | 49 12 3       | 12 3          | 3 3 3               |
| C13-EGFP      | 58 11 3      | 16 3 3        | 7 3 3         | 4 3 3               |
| CR1-EGFP      | 68 3 3       | 44 9 3        | 14" 4 3       | 4 3 3               |
| TrkB-EGFP     | 5 2 2        | 8 2 2         | 52 2          |                     |

*At 100 ng/ml BDNF the cell count was 46 ± 14 (n = 3), and at 200 ng/ml the cell count was 37 ± 5 (n = 3).*
ing that Ser324 and Asp326 may play an indirect role in TrkA specificity for NGF by influencing the conformation of segment C-E (which itself directly interacts with NGF). In addition, the buried residue at position 319 (Trp in TrkA and His in TrkC) was found to be important; it may influence the conformation of loop AB, loop EF (via Pro368), and the juxtamembrane segment (via Phe395 and other residues) (Fig. 3). Replacement of TrkC residues in loop AB and β-strand B with those of TrkA were required to recruit NGF binding but, notably, did not prevent NT-3 binding. This suggests that 1) loop AB and β-strand B provide for binding and specificity in the NGF/TrkA interaction but not in the NT-3/TrkC interaction and 2) NGF may be prevented from binding to TrkC due to the lack of appropriate residues in loop AB and β-strand B; indeed the TrkC residues in these segments might actually repulse NGF.

Since segment C-E differs in length and sequence among the Trks, the entire segment was exchanged instead of individual residues. NGF binding was optimal when segment C-E had the TrkA sequence (compare variants CR1 and CR2, Table II). In the crystal structure of the complex, residues in the C-terminal third of TrkA segment C-E interact with the NGF N terminus (36). As with loop AB and β-strand B, the presence of TrkA sequence in segment C-E did not hamper NT-3 binding (compare CR1 and CR2, Table II). This supports the contention that the NT-3 N-terminal residues are not important for interaction with TrkC.

The role of the juxtamembrane segment for Trk specificity has previously been noted from studies on natural variants of Trks in different cell types. One natural TrkA variant has an insert of six amino acids in the juxtamembrane segment (37), and a similar variant has been detected in TrkC (38). In fibroblasts, the presence of the TrkA insert did not affect ligand specificity; however, in PC12/nn5c cells the insert did affect specificity (39). Likewise, TrkB isoforms with differences in the juxtamembrane segment affect specificity (40, 41).

In this study, five residues in the N-terminal portion of the juxtamembrane segment were found to be required for NGF binding as follows: Met396, Asp397, Phe402, Glu405, and Asp406 (Table I and Fig. 1). The C-terminal residues, 408–419 (Fig. 1) were also required to be the TrkA sequence. In the crystal structure the methionine side chain is wedged between TrkA loop EF and NGF, interacting with hydrophobic side chains of the NGF. The interactions of residues beyond Pro399 (382 in TrkA numbering) cannot be assessed since these were disordered in the NGF/TrkA crystal structure (36). As noted in the crystal structure report, the Trk juxtamembrane segment may interact with neurotrophin loops 40–49 and 93–98; this is supported by previous data showing that exchanging these loop sequences between neurotrophins altered specificity (42–44).

Unexpectedly, both NGF and NT-3 preferred the TrkC sequence in the C-terminal portion of the juxtamembrane segment (compare CR1 and CR3, Table II). One possible explanation for this is that the TrkA residues present in CR3, including those in the N-terminal portion of the juxtamembrane segment, prevent the introduced TrkC sequence from adopting its native conformation. Alternatively, the presence of the TrkC sequence may disrupt the conformation of nearby segments, preventing binding of either NGF or NT-3.

Not only could NGF binding be recruited into a TrkC-based variant, but NT-3 binding was maintained. This implies that the residues dictating the specificity of TrkC for NT-3 and of TrkA for NGF are separate. A previous study mapped the TrkC-binding site for NT-3 to loop EF, one residue in loop AB (Glu319) and residue in β-strand G (His394), although the latter had only a minimal effect on binding (26). The same study, now substantiated by the crystal structure of the complex (36), showed that the TrkA-binding site for NGF is much larger and comprises loop EF and loop AB, as well as residues in β-strand B, segment C-E, the juxtamembrane segment, and the disulfide bond (26).

NT-3 interaction with TrkC is dominated by loop EF in TrkC (26). Since loop EF is conserved among the Trk receptors (except at two positions) (Fig. 1), NT-3 may be prevented from binding to TrkA due to repulsion by certain TrkA residues outside of loop EF, although this repulsion can be overcome at higher concentrations (10, 14, 31). NT-3 binding to variant C13 was reduced compared with variant CR1 (Table II). Likewise, BDNF can bind to CR1 but not C13 (Fig. 6). This suggests that residues that differ between CR1 and C13 are among those in TrkA that prevent binding by NT-3 and BDNF. These include His311 (loop AB), Arg334 (β-strand C), Glu367 (loop EF), several residues in β-strands F and G, as well as some in the juxtamembrane segment (Fig. 1). However, inspection of the NGF/TrkA crystal structure shows that only His311, Glu367, and the juxtamembrane segment make contact with NGF. Within the juxtamembrane segment, Met396 and Asp397 may contribute to the difference in binding between C13 and CR1; the involvement of individual residues beyond Asp397 cannot be discerned from the structure although they may play a significant role. Arg334 and residues in β-strands F and G are distant from the NGF/TrkA interface (36) and are unlikely to be specificity determinants.

Deciphering which residues in Trk receptors prevent binding of certain neurotrophins cannot be unambiguously ascertained from the crystal structure alone. Interaction between a Trk residue and a neurotrophin residue found in the crystal structure may not necessarily correlate with mutagenesis results. For example, in the NGF/TrkA crystal structure (36) TrkA Arg364 (347 in TrkA numbering) interacts with NGF Glu11, and one might expect this interaction to be important. However, in this study exchanging Arg364 for Leu364 had no effect on binding of NGF; in a previous study exchanging Arg364 → Ala in TrkA did not affect NGF binding and, likewise, exchanging Leu364 → Ala in TrkC did not affect NT-3 binding (26). Such discrepancies between crystal structures of hormone-receptor complexes and mutagenesis data have been noted previously (45).

In previous studies it was found that a relatively small number of residues in neurotrophins can be altered to generate neurotrophin variants with multiple specificity for Trk receptors. In NT-3 a single amino acid change, Asp15 → Ala, allowed binding to TrkB similar to that of BDNF binding to TrkB, while maintaining binding to TrkC (31). In NGF, changing five or six amino acids to NT-3 sequence provided binding both to TrkA and TrkC which was equivalent to native NGF and NT-3, respectively (46). These and other studies show that the neurotrophins may share conserved binding residues (e.g. Arg103) while having other residues involved in specificity. However, the amino acids in each neurotrophin which dictate specificity may not be identical. For example, the N terminus is important in NGF but not NT-3 and exchanging the residues in loop 40–49 between NGF and NT-3 altered specificity, whereas exchanging this loop between NGF and NT-4 did not alter specificity (44). The same situation is now apparent for the Trk receptors; they share a conserved binding motif (loop EF), have discrete sections involved in specificity (e.g. loop AB, β-strand B, and the juxtamembrane segment), and the importance of these may differ among the Trk receptors.

In a recent report, Barde and co-workers (47) found that association of the p75 neurotrophin receptor with TrkB could influence neurotrophin specificity in a transfected cell line. However, they also pointed out that they had previously shown
that natural TrkB variants in the extracellular juxtamembrane domain also show differences in specificity in the absence of p75 (40) and that there may be multiple ways by which selectivity is controlled. The goal of the present study was to elucidate the specificity of TrkA inherent in its amino acid sequence. In vitro protein/protein assays were employed so as to preclude complications due to putative selectivity/specificity influences in different cell types. Now that the primary specificity of TrkA is known, the relevance of this in different cellular contexts can be addressed.

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