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An immunoglobulin-like domain determines the specificity of neurotrophin receptors

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The neurotrophins influence survival and maintenance of vertebrate neurons in the embryonic, early postnatal and post-developmental stages of the nervous system. Binding of neurotrophins to receptors encoded by the gene family *trk* initiates signal transduction into the cell. trkA interacts preferably with nerve growth factor (NGF), trkB with brain-derived neurotrophic factor (BDNF) and neurotrophin-4/5 (NT-4/5) and trkC with neurotrophin-3 (NT-3). By constructing 17 different chimeras and domain deletions of the human trk receptors and analyzing their binding affinities to the neurotrophins we have shown that an immunoglobulin-like domain located adjacent to the transmembrane domain is the structural element that determines the interaction of neurotrophins with their receptors. Chimeras of trkC where this domain was exchanged for the homologous sequences from trkB or trkA gained high affinity binding to BDNF or NGF respectively, while deletion of this domain in trkC or trkA abolished binding to NT-3 or NGF respectively. This domain alone retained affinities to neurotrophins similar to the full-length receptors and when expressed on NIH 3T3 cells in fusion with the kinase domain showed neurotrophin-dependent activation.

Key words: binding domain/neurotrophins/trk/tyrosine kinase receptor

Introduction

Neurotrophins regulate the survival of neurons sensitive to their action during a period of naturally occurring cell death in embryonic life. Their action also extends into post-developmental stages of the nervous system, where they regulate the function of differentiated neurons (Snider, 1994). The highly homologous neurotrophin family includes nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and neurotrophin-4/5 (NT-4/5) (Barde, 1991). The signal transduction pathway of neurotrophins is initated by specific binding to the extracellular domain of receptor tyrosine kinases encoded by the *trk* gene family, which includes *trkA* (Martin-Zanca *et al.*, 1989), *trkB* (Klein *et al.*, 1989) and *trkC* (Lamballe *et al.*, 1991). Binding of neurotrophins leads to autophosphorylation of the receptors (Kaplan *et al.*, 1991), which is believed to be induced by receptor homodimerization, a common trigger for tyrosine kinase activation (Schlessinger and Ulrich, 1992). Autophosphorylation of trkA initiates its association with phospholipase C-γ1 and SHC, coupling the signaling pathway to phosphatidylinositol metabolism as well as to the regulation of $p21^{ras}$ activity (Obermeier *et al.*, 1994; Stephens *et al.*, 1994). These signals are propagated to other messengers and lead ultimately to differentiation and cessation of growth (Halegoua *et al.*, 1991).

Each trk receptor discriminates between the different neurotrophins (Figure 1A). While the trkA and trkC receptors bind and respond *in vitro* preferentially to NGF and NT-3 respectively (Kaplan *et al.*, 1991; Lamballe *et al.*, 1991), trkB interacts *in vitro* with BDNF, NT-4/5 and, to a lesser degree, with NT-3, but not with NGF (Ip *et al.*, 1992). Although NT-3 interacts *in vitro* with all three receptors to some extent, it has been suggested that the neuronal environment restricts the trk receptors in their ability to respond to non-preferred neurotrophin ligands (Ip *et al.*, 1993). By mutational analyses of human NT-3 (Urfer *et al.*, 1994) and human NGF (Ibáñez *et al.*, 1993; Shih *et al.*, 1994), the binding sites for trkC and trkA, respectively, were defined and mechanisms of specificity determination were proposed (Urfer *et al.*, 1994). In contrast, the structural elements of the trk receptors responsible for specific neurotrophin recognition remain undefined. Although the three-dimensional structure of the extracellular domain (ECD) of these receptors is unknown, a hypothesis delineating the domain organization was proposed based on sequence information (Schneider and Schweiger, 1991). According to this, the mature extracellular portion consists of a cysteine cluster (domain 1), a leucine-rich motif (domain 2) and a second cysteine cluster (domain 3) followed by two immunoglobulin-like domains (domains 4 and 5) (Figure 1B). Leucine-rich motifs flanked by cysteine clusters have been identified in many proteins (Kobe and Deisenhofer, 1994) and the presence of the two immunoglobulin-like domains in trk receptors has been proposed by Kuma *et al.* (1991) independently.

In this report the domain of human trk receptors responsible for specific binding to neurotrophins was determined by constructing a series of trk domain deletions and chimeras (domain exchanges between trkA, trkB and trkC), which were then analyzed for binding of the different neurotrophins and neurotrophin-dependent receptor activation. trkC chimeras where the immunoglobulin-

like domain closest to the transmembrane domain was exchanged for the homologous sequences from trkB or trkA bound BDNF and NGF respectively, with high affinity. Furthermore, deletion of this domain in trkC and trkA abolished binding to NT-3 and NGF respectively, whereas this domain alone bound with similar affinity to the neurotrophins as their respective full-length receptors. Hence, the second immunoglobulin-like domain not only confers specificity but provides the main contacts for binding of trk receptors to their ligands.

Results

Construction, expression and purification of receptor deletions and swaps

The design of deletions and chimeric receptors was dictated by the proposed position of the leucine-rich motif (Schneider and Schweiger, 1991), the consensus sequences for the cysteine clusters (Kobe and Deisenhofer, 1994) and the position of the immunoglobulin-like domains (Kuma *et al.*, 1991). Consequently, the first cysteine cluster extended from Cys1 to Arg47 (standard abbreviations for amino acids and numbering for mature human trkC; Shelton *et al.*, 1995), the leucine-rich motif from Asn48 to Asn131, the second cysteine cluster from Cys132 to Asn177, the first immunoglobulin-like domain from Leu178 to Val266 and the second immunoglobulin-like domain from Ala267 to Thr381.

A set of 17 trk receptor deletions and chimeric variants was constructed (Figure 1B). The designation of the receptor variants is as follows: ΔNy describes deletions (Δ) of single domains (N = 1–5) in a given receptor (y) (e.g. Δ2C means that the leucine-rich motif was deleted from the trkC receptor); sNy describes swaps (s) where individual domains from trkC (N = 1–5) were exchanged for homologous sequences from another trk receptor (y) (e.g. s5A is a chimeric trkC receptor which includes the second immunoglobulin-like domain from trkA). The cDNAs coding for individual domain deletions in trkC (Δ1C, Δ2C, Δ3C, Δ4C and Δ5C) were constructed by mutagenesis and flanking restriction sites which did not change the protein sequence were introduced simultaneously. PCR fragments of the homologous trkB domains, which carried compatible restriction sites at their 5′- and 3′-ends, were amplified and ligated to the corresponding trkC deletion variants, yielding trkC chimeras in which each of the five domains were individually exchanged for its trkB counterpart (s1B, s2B, s3B, s4B and s5B). Two additional trkC deletion mutants were constructed in which only the second immunoglobulin-like domain (closest to the transmembrane domain) was retained (Δ6C) or both immunoglobulin-like domains were retained (Δ7C). In order to extend our studies to the trkA/NGF system, we also constructed analogous deletions for trkA (Δ4A, Δ5A, Δ6A and Δ7A) and a trkC variant where the second immunoglobulin-like domain was exchanged for the homologous trkA sequence (s5A) (Figure 1B). All receptor variants were constructed as immunoadhesins in which the extracellular domains are fused to the F_c portion of a human antibody (Ashkenazi *et al.*, 1993). The extracellular domains of trkA, trkB and trkC expressed in this fashion have been shown to display the expected specificities (Shelton *et al.*, 1995) and to interact with high affinity with their respective neurotrophin ligands (Urfer *et al.*, 1994). In addition, it has been shown that a panel of NT-3 mutants displayed a similar profile of

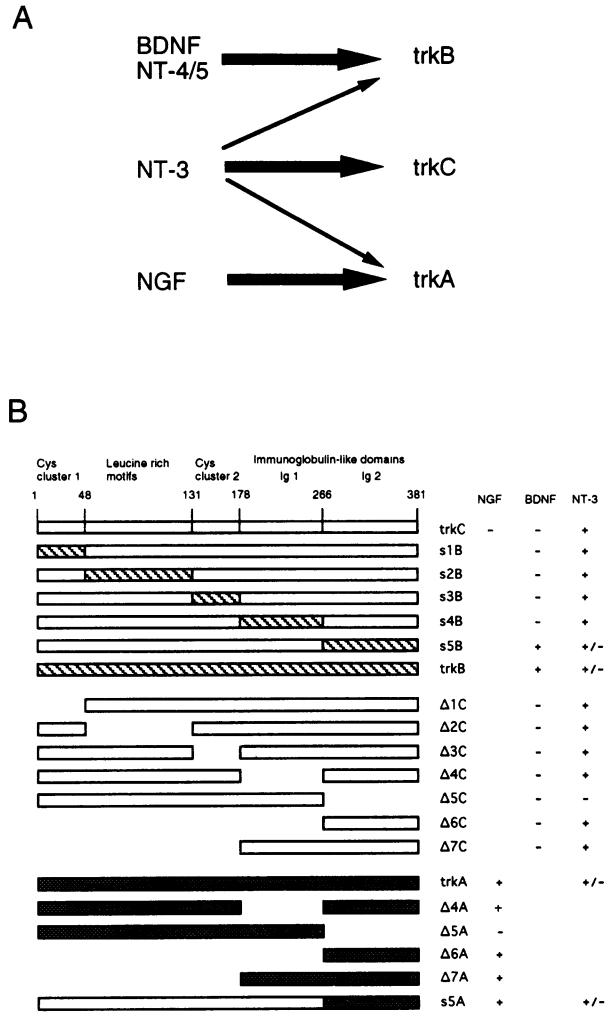

Fig. 1. Specificities of trk receptors and structural domain organization. (**A**) Specificities of neurotrophins for trk receptors. NT-3 primarily interacts with trkC, BDNF and NT-4/5 with trkB and NGF with trkA (thick arrows). NT-3 also binds with lower affinity to trkB and trkA (thin arrows). Note that trkC, the receptor for NT-3, does not interact with BDNF, NT-4/5 or NGF (for review see Chao, 1992). (**B**) Domain organization of trk receptors: cysteine cluster 1 (residues 1–47, numbering of residues starting with the first residue of the mature human trkC sequence), leucine-rich motif (residues 48–130), cysteine cluster 2 (residues 131–177), first immunoglobulin-like domain (residues 178–265), second immunoglobulin-like domain (residues 266–381). For chimeric receptors, domains derived from human trkC, trkB or trkA are depicted with open, hatched or closed bars respectively. In chimeras s1B–s5B each trkC domain was exchanged for the homologous trkB domain. In chimera s5A the second immunoglobulin-like domain from trkC was exchanged for the trkA sequence. The variants Δ1C–Δ5C and Δ4A and Δ5A are individual domain deletions of trkC and trkA respectively. The variants Δ6C and Δ6A have only the second immunoglobulin-like domain from trkC and trkA respectively. The variants Δ7C and Δ7A have the first and the second immunoglobulin-like domains from trkC and trkA respectively. The extracellular domain of all receptor variants was fused to the F_c portion of a human antibody and expressed as an immunoadhesin (Ashkenazi *et al.*, 1993). The binding characteristics for each of the receptor variants are qualitatively shown to the right. Affinities of receptors for NGF, BDNF and NT-3 are compared with trkA, trkB and trkC respectively: (+) $IC_{50mut}/IC_{50wt} < 3$; (+/–) $40 > IC_{50mut}/IC_{50wt} > 8$; (–) no binding detected.

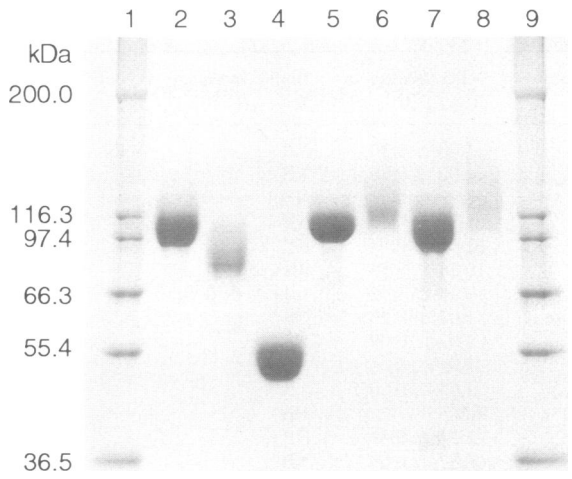

Fig. 2. Expression and purification of receptor variants. All variants were expressed as immunoadhesins and purified to homogeneity by protein A affinity chromatography. Proteins are shown after SDS–PAGE on an 8–16% gel (Novex) under reducing conditions and stained with Coomassie blue. Molecular weights (kDa) of standard proteins are indicated (lanes 1 and 9), trkC (lane 2), Δ5C (lane 3), Δ6C (lane 4), trkB (lane 5), s5B (lane 6), trkA (lane 7) and s5A (lane 8). The calculated molecular weights for trkC, Δ5C, Δ6C, trkB, s5B, trkA and s5A are 68.5, 55.2, 39.2, 68.9, 71.4, 66.0 and 69.3 kDa respectively. The higher apparent molecular weights indicate significant glycosylation of the expressed receptors. Receptor mutants shown were blotted onto PVDF membranes by Western transfer for N-terminal sequencing. All variants had the expected N-terminal sequences (data not shown).

affinities for trkC whether expressed as immunoadhesin or on NIH 3T3 cells (Urfer *et al.*, 1994). This indicates that the binding domains of trkC have a similar conformation in both systems. The proteins were expressed in human 293 cells and purified as described in Materials and methods. The immunoadhesins, as shown for trkC, Δ5C, Δ6C, trkB, s5B, trkA and s5A (Figure 2), were >95% pure as judged from SDS–PAGE. The apparent molecular weights indicate significant amounts of glycosylation, which amounted to ~26 kDa for trkC and ~20 kDa for trkB. Similar values (28.5 kDa for trkC and 23.8 kDa for trkB) were determined by sedimentation equlibrium measurements (Philo *et al.*, 1994). In addition, the amount of glycosylation for Δ5C was estimated to be ~23 kDa, indicating that most of the glycosylation occurs in the first four domains. The N-terminal sequences were determined after Western blotting and resulted in the expected sequences (data not shown).

The second immunoglobulin-like domain determines the ligand specificity of neurotrophin receptors

trkC binds with high affinity to NT-3 but not to BDNF and NGF (Lamballe *et al.*, 1991; Tsoulfas *et al.*, 1993) and represents the most stringent member of the trk family with regard to its ligand specificity (Figure 1A). In contrast, its preferred ligand NT-3 also binds with reduced affinity to trkB and trkA (Urfer *et al.*, 1994). trkB on the other hand binds both BDNF and NT-4/5, but also with lower affinity to NT-3. If trkC and trkB use the same structural elements for ligand binding, recruitment of BDNF binding in a particular trkC/trkB chimera would be expected to be accompanied by a reduction in (but not complete loss of) NT-3 binding affinity. Similarly, recruitment of NGF binding in a trkC/trkA chimera would be paralleled by a reduction in NT-3 binding. Therefore, chimeras were assayed for both recruitment of binding affinity for BDNF (s1B, s2B, s3B, s4B, s5B) or NGF (s5A) and remaining NT-3 affinity.

In competitive displacement assays with BDNF, the trkB receptor displayed an IC_{50} of 20.7 ± 2.8 pM, while trkC did not bind BDNF (Figure 3A). In the chimera s5B the second immunoglobulin-like domain from trkC was exchanged for the homologous sequence from trkB. This chimeric receptor bound BDNF with an IC_{50} of 16.7 ± 1.5 pM (Figure 3A), an affinity very similar to the trkB receptor (Table I). Saturation binding experiments with [^{125}I]BDNF confirmed these observations; trkB and s5B showed similar K_d values of 10.4 ± 1.6 and 10.9 ± 2.2 pM respectively (Table II). None of the other trkC/trkB chimeras showed BDNF binding and domain deletions of trkC did not recruit any BDNF affinity (Table I). The latter observation excludes the possibility of a repulsive effect on BDNF by individual trkC domains. Hence the information necessary to change the trkC specificity from NT-3 to BDNF resides solely in the second immunoglobulin-like domain of trkB.

In order to determine whether structural elements present on trkB could possibly prevent binding of NT-3, all chimeras, as well as the native receptors, were assayed for NT-3 affinity. In agreement with earlier results where trkB was expressed on NIH 3T3 cells (Escandón *et al.*, 1994), the trkB receptor immunoadhesin bound NT-3 with an affinity reduced by 11.0 ± 1.7-fold compared with trkC (Table I). An identical loss of affinity of the s5B variant for NT-3 (10.3 ± 2.6-fold) was observed. Together with the unreduced affinities of s1B, s2B, s3B and s4B (<1.5-fold) (Table I), these data suggest that domains 1–4 of the trkB receptor contain no elements which prevent NT-3 binding or participate in ligand discrimination. Hence the specificity of the trkC and trkB receptors for NT-3 versus BDNF resides solely in the second immunoglobulin-like domain.

In order to probe further the importance of this domain for specific neurotrophin binding we constructed a trkC chimera (s5A) in which this domain was substituted by its trkA counterpart (Figure 1B). Competitive displacement assays showed that trkA, but not trkC, binds NGF with high affinity (Figure 3B). The s5A chimera recruited NGF binding (IC_{50} 39.3 ± 1.7 pM), displaying a similar affinity for NGF as trkA (IC_{50} 73.9 ± 8.1 pM) (Figure 3B). Saturation binding experiments with [^{125}I]NGF resulted in K_d values of 47.1 ± 12.4 and 38.6 ± 8.6 pM for trkA and s5A respectively (Table II), establishing the importance of this domain for ligand recognition in the NGF/trkA system. The similarity of the affinities of trkA and s5A also implies that there are no additional structural elements on trkC repulsive to NGF. The trkA receptor had a 36.1 ± 10.0-fold reduced affinity for NT-3 when compared with trkC, while the affinity of the s5A variant was reduced only 8.0 ± 0.4-fold (Table I). This observation suggests that there are additional elements in domains 1–4 of trkA which prevent binding to NT-3 to some extent.

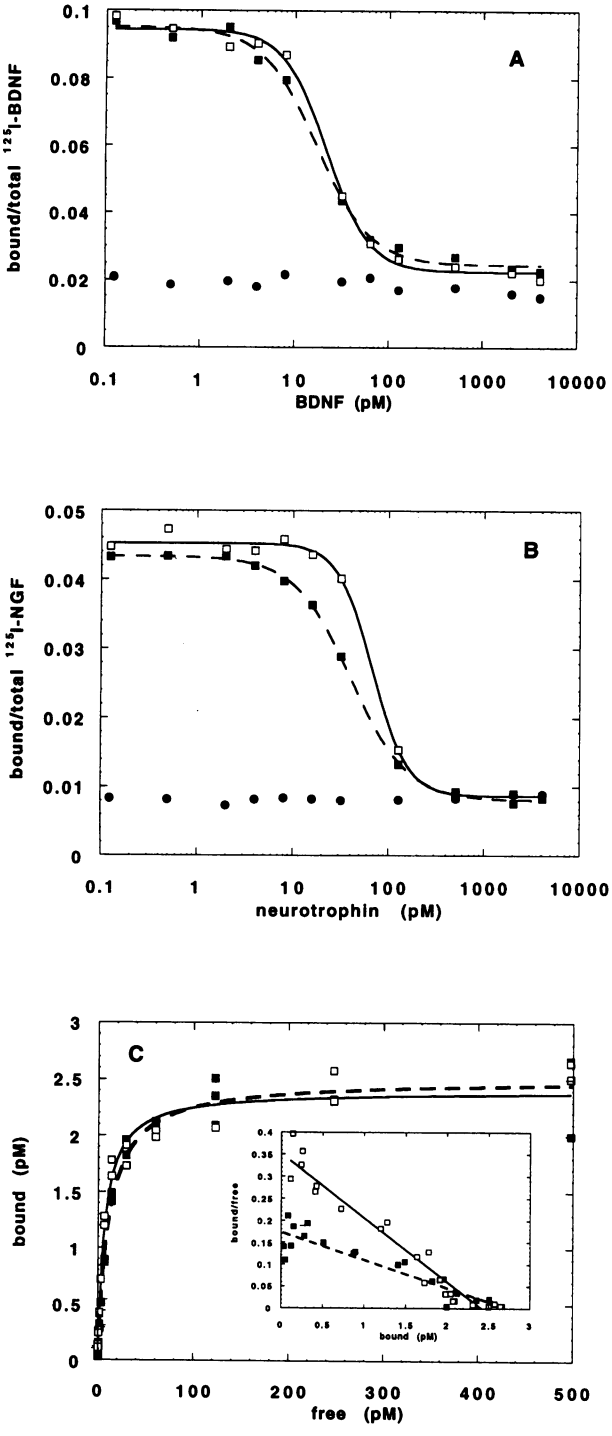

Fig. 3. The second immunoglobulin-like domain determines specificity and general binding of trk receptors. (**A**) Binding of BDNF to trkB (open squares), s5B (closed squares) and trkC (closed circles). All other trkC deletions and chimeras displayed binding traces equivalent to the one shown for trkC. Similar results were obtained in three independent experiments. The IC_{50} values for trkB and s5B were 20.7 ± 2.8 and 16.7 ± 1.5 pM respectively. (**B**) Binding of NGF to receptor trkA (open squares), s5A (closed squares) and trkC (closed circles). Similar results were obtained in three independent experiments. The IC_{50} values for trkA and s5A were 73.9 ± 8.1 and 39.3 ± 1.7 pM respectively. (**C**) Saturation binding of NT-3 to trkC and the Δ6C variant. Purified trkC (open squares) and Δ6C (closed squares) were incubated with increasing concentrations of [^{125}I]NT-3 and specific binding was determined as described in Materials and methods. The inset shows the Scatchard transformation of the saturation binding data. All saturation experiments were performed at least three times and resulting K_d values are shown in Table II.

Table I. Competition binding of neurotrophin receptor variants

Receptor	NT-3 binding IC_{50mut}/IC_{50trkC}	BDNF binding $IC_{50mut}/IC50_{trkB}$	NGF binding $IC50_{mut}/IC_{50trkA}$
trkC	1.00 ± 0.10	NB	NB
s1B	1.02 ± 0.10	NB	–
s2B	1.23 ± 0.22	NB	–
s3B	1.39 ± 0.26	NB	–
s4B	1.05 ± 0.04	NB	–
s5B	10.28 ± 2.55	0.81 ± 0.04	–
trkB	10.98 ± 1.72	1.00 ± 0.13	–
Δ1C	1.07 ± 0.26	NB	–
Δ2C	1.02 ± 0.09	NB	–
Δ3C	1.04 ± 0.06	NB	–
Δ4C	1.04 ± 0.06	NB	–
Δ5C	NB	NB	–
Δ6C	1.34 ± 0.34	NB	–
Δ7C	0.87 ± 0.06	NB	–
trkA	36.10 ± 10.00	–	1.00 ± 0.05
s5A	8.0 ± 0.4	–	0.54 ± 0.08
Δ4A	–	–	2.25 ± 0.34
Δ5A	–	–	NB
Δ6A	–	–	1.21 ± 0.20
Δ7A	–	–	1.04 ± 0.05

Receptor variants as shown in Figure 1. Results are expressed as the average of at least three independent binding experiments ± SD. NB means that no specific binding of ligand was detected under the conditions described in Materials and methods. –, not determined.

Table II. Saturation binding of neurotrophin receptor variants

Receptor	Ligand	K_d (pM)
trkC	[^{125}I]NT3	8.4 ± 1.6
Δ5C	[^{125}I]NT3	NB[a]
Δ6C	[^{125}I]NT3	20.9 ± 10.2
trkB	[^{125}I]BDNF	10.4 ± 1.6
s5B	[^{125}I]BDNF	10.9 ± 2.2
trkA	[^{125}I]NGF	47.1 ± 12.4
s5A	[^{125}I]NGF	38.6 ± 8.6
Δ5A	[^{125}I]NGF	>3500[b]
Δ6A	[^{125}I]NGF	155.3 ± 33

Results are expressed as the average of three independent binding experiments ± SD. K_d values were determined as described in Materials and methods. Specific binding was calculated as total binding minus binding in the presence of a 100-fold excess of unlabeled neurotrophin.
[a]Specific binding accounting for <6% of the total binding was detectable at high ligand concentrations.
[b]Saturation was not reached and therefore the K_d value shown is an estimate for the lower limit.

The isolated second immunoglobulin-like domain of trkC and trkA displays high affinity binding to neurotrophins

The trk chimeras elucidated the importance of the second immunoglobulin-like domain for ligand discrimination in the trkC/trkB and trkC/trkA systems, but did not determine the contribution of individual domains to the general affinity for neurotrophins. To address this, each of the domains of trkC was deleted individually and the resulting proteins were assayed for NT-3 binding. The deletion variants Δ1C, Δ2C, Δ3C and Δ4C bound NT-3 with similar affinities as the full-length trkC (Table I), thereby excluding an involvement of domains 1–4 in high affinity binding to NT-3. In contrast, variant Δ5C (which has the second immunoglobulin-like domain deleted) did not show detectable NT-3 binding in competitive displacement assays

(Table I). Saturation binding experiments showed only a low amount of specific bound NT-3 at high ligand concentrations, which accounted for <6% of the total bound ligand (Table II). In order to determine whether this domain alone would be sufficient for high affinity NT-3 binding, an additional variant Δ6C was constructed in which domains 1–4 were deleted (i.e. only the second immunoglobulin-like domain was retained). This variant bound NT-3 with almost identical affinity (IC_{50} 25.6 ± 0.5 pM) as trkC (IC_{50} 18.9 ± 0.8 pM); Δ6C did not bind BDNF, as expected (Table I). In saturation binding experiments the K_d values for Δ6C and trkC were 20.9 ± 10.2 pM and 8.4 ± 1.6 pM respectively (Figure 3C and Table II). The reduction in NT-3 binding by Δ6C is only 2.5-fold, suggesting that this trkC domain alone provides most of the binding affinity for NT-3. When both immunoglobulin-like domains are present (variant Δ7C), NT-3 binding is only slightly improved compared with Δ6C (Table I). This may be due to the presence of some minor binding elements in the first immunoglobulin-like domain or this domain may help stabilize the fold of the second immunoglobulin-like domain.

Analogous deletions of the trkA receptor were assayed for NGF binding in competitive displacement assays. Similar to the results obtained from the trkC receptor variants, deletion of the second immunoglobulin-like domain in variant Δ5A resulted in no detectable NGF binding, while this domain alone (variant Δ6A) retained binding affinity for NGF (Table I). In addition, deletion of the first immunoglobulin-like domain (Δ4A) reduced the affinity only 2.3-fold and deletion of the first three domains (Δ7A) had no influence on affinity for NGF (Table I). When the NGF affinities of trkA and Δ6A were determined in saturation experiments the K_d values were 47.1 ± 12.4 and 155.3 ± 33 pM (a 3.3-fold difference), verifying that in the trkA receptor most of the binding interaction with NGF is provided by the second immunoglobulin-like domain (Table II). However, saturation experiments with the Δ5A variant resulted in detectable specific binding, with an estimated K_d value of >3500 pM (Table II), indicating the possible presence of additional elements in trkA domains 1–4 that interact with NGF, although their contribution to binding seems to be minor, as evidenced by the similar K_d values of the Δ6A variant and trkA.

Neurotrophin-induced autophosporylation of receptor variants

In order to test the ligand interactions of the different receptor variants in a cellular environment, stable NIH 3T3 cell lines expressing either trkC, s5B, trkB, Δ5C, Δ6C or Δ7C were constructed as described in Materials and methods. trkC and all trkC variants contained the transmembrane and intracellular domains from trkC. At least three independent cell lines for each of the constructs were selected and assayed for neurotrophin-induced autophosphorylation. The expression of the receptors was demonstrated by immunoprecipitation with the pan-trk antibody 443 (Martin-Zanca *et al.*, 1989) (Figure 4). As reported for the rodent trk receptors (Klein *et al.*, 1991; Meakin *et al.*, 1992; Tsoulfas *et al.*, 1993), the apparent molecular weights of the full-length human trkC and trkB receptors indicate the presence of a significant amount of

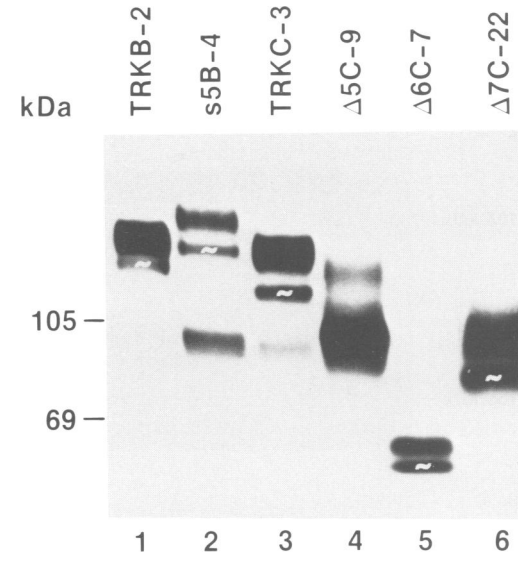

Fig. 4. NIH 3T3 cell lines expressing receptor variants. The extracellular domains of trkC, s5B, Δ5C, Δ6C and Δ7C were fused to the transmembrane and intracellular domains of trkC and stably expressed on NIH 3T3 cells, while the trkB cell line expressed the full-length human trkB receptor. The presence of receptors is shown after immunoprecipitation with the pan-trk 443 antiserum. ~ marks a precursor as previously described for rat and porcine trk receptors (Lamballe *et al.*, 1991; Tsoulfas *et al.*, 1993). Note that s5B runs at a higher apparent molecular weight than trkB due to its longer amino acid sequence. The smaller bands (~105 kDa) in lanes 2 and 3 may represent degradation products of the s5B and trkC receptors. At least three independent cell lines were selected for each of the constructs and assayed for neurotrophin-induced autophosphorylation. All trkC receptor variants cross-reacted with the trkC-specific 656 antibody, while trkB displayed a signal with a trkB-specific antibody (data not shown).

glycosylation. In addition to the predominant larger band, all NIH 3T3 cells expressed a smaller protein species (marked with ~) which was immunoprecipitable by the pan-trk 443 antiserum. This observation is in agreement with earlier reports on expression of porcine and rat trkC in NIH 3T3 cells (Lamballe *et al.*, 1991; Tsoulfas *et al.*, 1993) and the band may represent a partially glycosylated precursor. The variant Δ5C has a calculated molecular weight of 104 kDa, including 23 kDa glycosylation, which agrees well with the apparent molecular weight in Figure 4. In addition, Δ6C and Δ7C run at lower positions than trkC, as expected, due to their reduced molecular weights (Figure 4). The cell lines expressing trkC and s5B also exhibited to some extent a degradation product of ~105 kDa; nevertheless a significant portion was expressed with the expected molecular weights for the full-length receptors. The immunoprecipitated receptors from trkC, s5B, Δ5C, Δ6C and Δ7C as shown in Figure 4 originated from similar numbers of cells, while for trkB 4-fold fewer cells were processed. Receptors from all cell lines were run on the same gel and all lanes were simultanously exposed to film. Only the trkB-expressing cell lines reacted with a trkB-specific antiserum (data not shown) and all trkC receptor variants were recognized by the trkC-specific antiserum 656 (data not shown). Since the pan-trk antiserum 443 recognizes the C-terminal end of the kinase domain, the presence of a signal indicates that all receptors are expressed to full length and remain unprocessed at their C-termini.

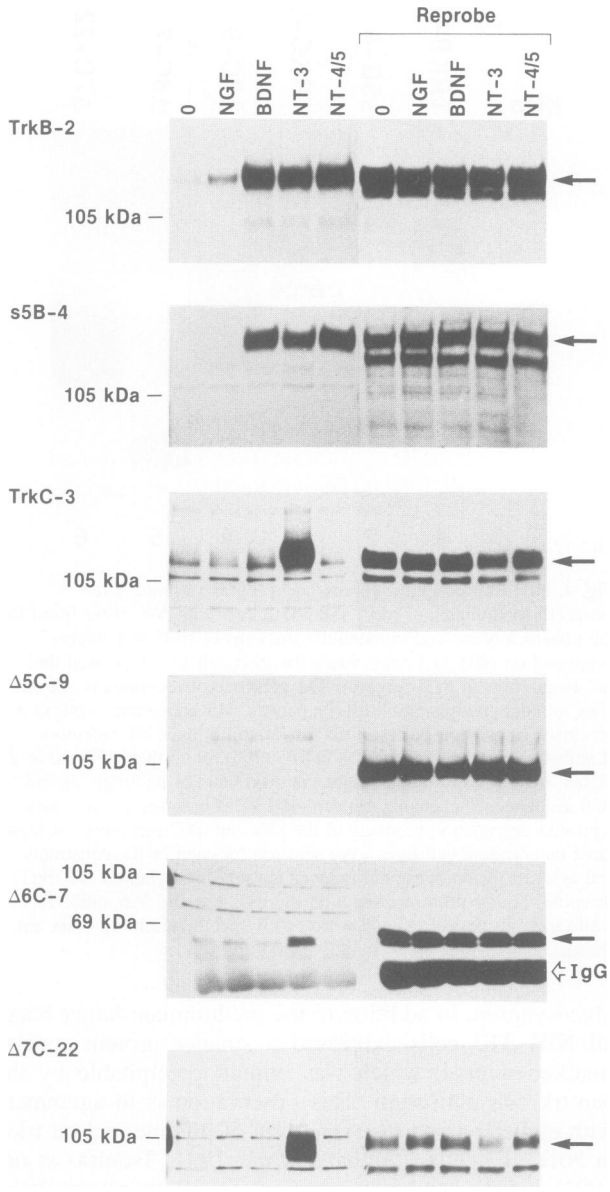

Fig. 5. Neurotrophin-induced autophosphorylation of receptor variants. NIH 3T3 cell lines expressing full-length trkB (panel 1, TrkB-2), s5B (panel 2, s5B-4), TrkC (panel 3, trkC-3), Δ5C (panel 4, Δ5-9), Δ6C (panel 5, Δ6-7) or Δ7C (panel 6, Δ7-22) were stimulated with no neurotrophin or 100 ng/ml NGF, BDNF, NT-3 or NT-4/5. The receptors were immunoprecipitated with the pan-trk antiserum 443 and bands were stained with an anti-phosphotyrosine antibody 4G10 after Western transfer as described (left panels; Soppet *et al.*, 1991; Tsoulfas *et al.*, 1993). Re-probing of the blots with the pan-trk 443 antiserum (right panels) demonstrated that equal levels of receptors were loaded for each of the receptor variants for the different neurotrophin-induced autophosphorylations. Arrows depict positions of trk receptor variants. IgG in panel 5 shows migration of the rat 443 antibody used for immunoprecipitation. This band appears on all other panels, but is only visible in panel 5 due to the smaller size of the Δ6 variant.

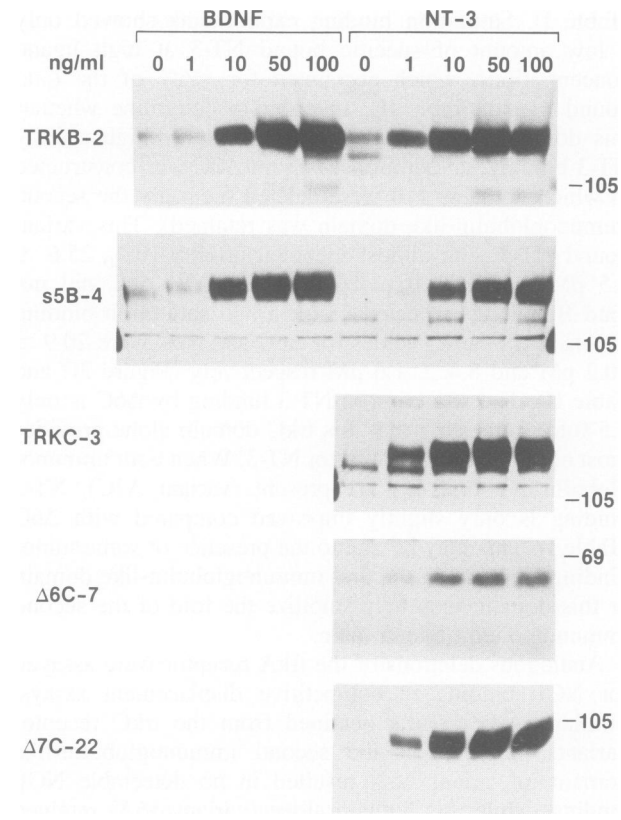

Fig. 6. Dose-dependency of neurotrophin-induced autophosphorylation of receptor variants. NIH 3T3 cell lines expressing full-length trkB (panel 1, TRKB-2) or s5B (panel 2, s5B-4) were stimulated with 0, 1, 10, 50 or 100 ng/ml BDNF or NT-3. Cell lines expressing trkC (panel 3, TRKC-3), Δ6C (panel 4, Δ6-7) or Δ7C (panel 5, Δ7-22) were stimulated with 0, 1, 10, 50 or 100 ng/ml NT-3. The receptors were immunoprecipitated with the pan-trk antiserum 443 and bands were detected with the anti-phosphotyrosine antibody 4G10 after Western transfer.

The profiles of neurotrophin-induced autophosphorylation of these cell lines are shown in Figure 5. Rat trkB when expressed on NIH 3T3 cells becomes autophosphorylated upon addition of BDNF, NT-4/5 and NT-3, but not by NGF (Ip *et al.*, 1993). A similar profile of activation was observed for human trkB (Figure 5, panel 1), where BDNF, NT-4/5 and NT-3 induced strong autophosphorylation, while NGF showed only a weak signal above background when assayed at 100 ng/ml. Conversely, rat trkC expressed on NIH 3T3 cells becomes autophosphorylated exclusively by NT-3 (Tsoulfas *et al.*, 1993). The same was observed for human trkC, where NT-3 (but not NGF, BDNF or NT-4/5) induced rapid autophosphorylation (Figure 5, panel 3). When the cell line expressing s5B was assayed, autophosphorylation was induced by NT-3, BDNF and NT-4/5, but not NGF, establishing an activation profile similar to trkB (Figure 5, panel 2). In order to ensure that the number of cells was constant within each of the autophosphorylation assays, the blots on the left side of Figure 5 were stripped of the anti-phosphotyrosine antibody (4G10) and re-probed with the pan-trk 443 antibody. As shown in the right panels of Figure 5, the amount of receptor within each cell line was constant for each of the neurotrophin-induced autophosphorylations; therefore the absence of signal can be attributed to non-activation of the receptor. In agreement with the established binding affinities of s5B to BDNF and NT-3, the dose dependency of BDNF- and NT-3-induced autophosphorylation was similar to trkB (Figure 6, panels 1 and 2) and showed saturation at 50 ng/ml for both BDNF and NT-3. These observations are in agreement with results obtained with neurotrophin-induced autophosphorylation of rat trkB, where saturation was reached between 50 and 250 ng/ml (Soppet *et al.*, 1991). Hence the s5B chimera

represents a trkC variant that exhibits BDNF- and NT-4/5-dependent autophosphorylation, although it carries most of the ECD, the entire transmembrane and the entire cytoplasmic domains from trkC.

Cell lines expressing domain deletions of the trkC receptor were analyzed for neurotrophin-induced autophosphorylation. As expected from the binding studies, autophosphorylation was not detected for variant Δ5C (Figure 5, panel 4). Re-probing of the blot with the pan-trk 443 antiserum showed that the receptor was expressed and that the lack of autophosphorylation could be attributed to a lack of interaction with neurotrophins. Most importantly, variant Δ6C when expressed on NIH 3T3 cells showed low but significant and specific induction of autophosphorylation by NT-3, but not NGF, BDNF or NT-4/5 (Figure 5, panel 6). Therefore, in addition to being essential for specific binding to NT-3, this domain also induces neurotrophin-dependent autophosphorylation. No signal above background was observed for NGF, BDNF or NT-4/5, demonstrating the maintenance of specificity by Δ6C. Re-probing of the blot revealed that the expression level of Δ6C was reduced compared with full-length receptor (Figure 5, panels 3 and 5) and therefore the reduced autophosphorylation signal may in part be due to the lower expression of Δ6C. None of the other cell lines tested expressed the Δ6C variant at higher levels (data not shown). Dose–response experiments showed that this variant receptor reaches saturation at 50 ng/ml, similar to the full-length trkC receptor (Figure 6, panel 4). Autophosphorylation of Δ6C does not rule out the possibility that additional elements may be required for efficient activation of the trkC receptor. When the first and the second immunoglobulin-like domains were expressed together in variant Δ7C, a response to NT-3 comparable with trkC was found (Figure 5, panel 6 and Figure 6, panel 5). This observation suggests that the first immunoglobulin-like domain may be involved in efficient expression and folding of the second immunoglobulin-like domain and/or may help in the formation of a stable signal transducing complex.

Discussion

A considerable amount of evidence has established the importance of trk receptor tyrosine kinases in transducing the signal of neurotrophins into the cell (reviewed in Kaplan and Stephens, 1994). The trk receptors form a family of homologous proteins, as do the neurotrophins, and though the different members within these two families probably have similar structures, they interact with each other in a very specific manner. In this study the domain of trk receptors responsible for specific recognition of neurotrophins was identified by constructing and analyzing a variety of receptor variants. These included domain deletions of trkC and trkA, as well as chimeras in which domains from trkC were exchanged for the homologous sequences from trkB or trkA. The results showed that the second immunoglobulin-like domain (that closest to the transmembrane domain) is the element that determines the interaction of each trk receptor with its specific neurotrophin. A trkC variant in which this domain was exchanged for the homologous sequence from trkB bound BDNF with high affinity and when this chimera was expressed on NIH 3T3 cells in fusion with the trkC intracellular domain, BDNF induced autophosphorylation. Similarly, a trkC variant in which this domain was exchanged for the homologous sequence from trkA bound NGF with high affinity. Deletion of this domain in trkC or trkA abolished binding to NT-3 and NGF respectively. When expressed in the absence of other domains, the second immunoglobulin-like domains of trkC and trkA retained affinities for neurotrophins similar to the respective full-length receptors.

Comparison of the trk receptor sequences between different species, as well as between the members within a given species, revealed that the degree of conservation of the second immunoglobulin-like domain is above the average calculated for all domains (Shelton *et al.*, 1995). This may be linked to its important role in receptor function. The binding domain contains two highly conserved segments between residues Cys320 and Gly339 and Gly361 and Leu378 (trkC numbering) with amino acid identities of 55 and 67% respectively, among all species and members of the trk family. Each of the segments includes one of the cysteines that are conserved in all trk receptors and the second segment has been proposed to be involved in trkC receptor signaling (Lamballe *et al.*, 1991). A complete mutational analysis of this domain will give insight into the relative contributions of these regions to specific binding of neurotrophins. This information can be combined with the results from the mutational analyses of NGF and NT-3 (Ibáñez *et al.*, 1993; Shih *et al.*, 1994; Urfer *et al.*, 1994) to yield a more precise knowledge of the interactions between trk receptors and neurotrophins. Comparison of the neurotrophin epitopes involved in binding to the trks revealed that both NGF and NT-3 make use of similar residues in the β-strand bundle. In addition, the NGF binding site for trkA includes the six N-terminal and certain loop residues which are not involved in binding of NT-3 to trkC. This suggests that the surface of interaction with the receptor is larger in NGF than it is in NT-3 and therefore it is conceivable that the trkA receptor also uses a larger surface to interact with neurotrophins than does trkC. Supporting evidence for this is provided by the observations that elements in domains 1–4 of trkA prevent binding to NT-3 to some extent and that deletion of the second immunoglobulin-like domain in trkA did not abolish binding to NGF completely.

The expression of full-length trks on NIH 3T3 cells leads to high and low affinity binding sites for neurotrophins (Soppet *et al.*, 1991, Jing *et al.*, 1992, Tsoulfas *et al.*, 1993). Although the molecular nature of the two classes of binding sites is unknown, it was proposed that tyrosine protein kinase receptors exist in a dynamic equilibrium between monomeric (low affinity) and dimeric (high affinity) structures, with only the latter being responsible for mediating signal transduction (Jing *et al.*, 1992). The tyrosine phosphorylation of trkB by BDNF or trkC by NT-3 with half maximal responses at 10 ng/ml (Figure 6) suggests affinities of ~400 pM. Conversely, the amount of neurotrophic factors needed to elicite half-maximal responses for survival of cultured neurons is at least 100-fold lower (Davies *et al.*, 1993). Therefore, formation of an active signaling complex of trks is somehow favored in neurons compared with NIH 3T3

cells. Similarly, the high affinity binding of neurotrophins to the trk immunoadhesins may be due to a shift in the equilibrium between monomeric and dimeric forms of the receptor, forced by the dimeric nature of the constant domains of the human antibody to which the receptors are fused. An alternative explanation for the observed differences in the potency of neurotrophic effects on neurons and NIH 3T3 cells may be that the amount of signal per cell necessary to elicite an effect may be very low for neurons (pM in terms of neurotrophin concentration) and therefore the observed low potency of neurotrophins in inducing trk receptor responses in NIH 3T3 cells may not fully reflect the *in vivo* situation.

The determination of the three-dimensional structures of NGF (McDonald *et al.*, 1991), PDGF (Oefner *et al.*, 1992) and TGF-β (Schlunegger and Grütter, 1992) revealed that all three molecules share a common motif referred to as the cysteine knot (McDonald and Hendrickson, 1993). In addition to the unique arrangement of disulfide bonds, these growth factors adopt similar structures with respect to the arrangement of secondary structure elements. Consequently, these otherwise unrelated growth factors were proposed to form a structural superfamily (McDonald and Hendrickson, 1993). Mutational analysis of the α-PDGF receptor showed that it uses immunoglobulin-like domains to interact with its ligand (Heidaran *et al.*, 1990). The present study has demonstrated that the neurotrophins also bind and activate their receptors by specific interaction with an immunoglobulin-like domain. If these growth factors evolved from a common ancestor by divergent evolution, it seems that not only their three-dimensional structure was conserved but also the structural domain they use for mediating their function. Alternatively, these factors may have evolved to a similar structure by convergence and the observation that they interact with immunoglobulin-like domains may be due to a favorable complementarity between these particular folds.

Since specific binding of neurotrophic factors is determined by a single domain, the function of the other domains remains unclear. The second immunoglobulin-like domain is sufficient for specific high affinity binding of neurotrophins and activation of the receptor does occur when this domain is expressed alone on NIH 3T3 cells. However, this domain was not as highly expressed on NIH 3T3 cells as the full-length receptor and therefore additional domains may be required for efficient expression, *in vivo* folding and translocation across the membrane. In addition, autophosphorylation may not be as efficient as when the full-length receptor is stimulated, suggesting that additional elements (possibly the first immunoglobulin-like domain) may help in the formation of a stable signal transducing complex by controling receptor dimerization. In this respect it is interesting to note that a constitutively active form of trk, the trk5 oncogene, carries a 51 amino acid deletion which is located within the second immunoglobulin-like domain (Coulier *et al.*, 1990). Although the mechanism of oncogenic activation remains unknown, these results suggest that domains other than the second immunoglobulin-like domain may contribute to the formation of a stable signalling complex. Alternatively, removal of a large part of the neurotrophin binding domain will likely change the conformation of other domains. This new conformation could facilitate dimerization of domains which would normally not interact and therefore lead to constitutive activation in the absence of the ligand.

Cell adhesion molecules (CAM, e.g. L1) are thought to play an important role in the development and maintenance of the nervous system by promoting morphological plasticity and maintaining stable contacts between cells (reviewed in Goodman and Shatz, 1993). A soluble L1–F_c chimera could effectively stimulate neurite outgrowth from primary neurons by directly or indirectly activating neuronal fibroblast growth factor receptors (Doherty *et al.*, 1995). In analogy, it is conceivable that trk receptors carry CAM binding domains that are likely to be distinctive from the neurotrophin binding domain. A candidate for this function is the leucine-rich motif, since all leucine-rich repeat containing proteins appear to be involved in protein–protein interactions and at least half of them take part in signal transduction pathways (Kobe and Deisenhofer, 1994). Finally, earlier functional studies have reported that neurotrophins bound to trk receptors are internalized and retrogradely transported to the cell body (DiStefano *et al.*, 1992). Hence, an alternative function for the trk receptor domains not used in binding might involve attachment to transport proteins within the cell. Future experiments will involve expression of the trks and trk variants in PC12 cells as well as in neuronal cells in order to gain insights into the physiological functions of the domains not involved in neurotrophin binding.

Materials and methods

Mutagenesis and recombinant DNA manipulations

Immunoadhesins of the extracellular domains of human neurotrophin receptors trkA, trkB and trkC were previously constructed, sequenced and subcloned into a vector which allows for production of double- and single-stranded DNA in *Escherichia coli*, as well as expression of mature receptors in a mammalian system under control of the cytomegalovirus promoter (Shelton *et al.*, 1994). Mutagenesis on this vector was performed according to the method of Kunkel (Kunkel, 1985). After transformation into the *E.coli* strain XL1-Blue, colonies were screened for the presence of the desired mutation by sequencing single-stranded DNA using the Sequenase version 2.0 kit (US Biochemical Corp.). For each selected clone, the entire sequence coding for the mature receptor–immunoadhesin was verified. Double-stranded DNA used for transfection of 293 cells was isolated from XL-1 Blue with the QIAGEN DNA purification kit (Qiagen Inc., Chatsworth, CA).

Design and construction of receptor variants

Structural and sequence considerations dictated the position of the domain boundaries. Once these positions were defined, deletion mutants were constructed which removed the particular domain and introduced flanking in-frame restriction sites. In order to create chimeras, the desired domain fragment was amplified using PCR primers which contained the same flanking restriction sites in-frame with the sites created during the deletion mutagenesis. Cutting the deletion mutant plasmid as well as the PCR fragment with the restriction enzymes and subsequent ligation of the fragments yielded plasmids coding for the desired chimeras.

For the deletion of domain 1 of trkC (Δ1C), the oligonucleotide 5′-CCAGTTCTCTATGTGTATACTAGTGATATTAGCCAGCACGG-AGC-3′ was used to loop-out amino acids Cys1–Arg47 (human trkC numbering starting with the first residue after the signal sequence). The corresponding trkC/trkB chimera s1B was constructed by site-directed mutagenesis of the trkC domain 1.

For Δ2C the oligonucleotide 5′-CATCCAGCGGATATCACAGCTGC-AATTTCTAGAGATGTCCGTGATG-3′ was used to loop-out amino acids Ile49–Asn132 and introduce an *Xba*I site and an *Eco*RV site at the 5′- and 3′-ends of the domain respectively. The primers used to amplify trkB domain 2 were 5′-CAGGTCAATCTCTAGAAACATCA-

CCGAAATTTTC-3′ and 5′-GGTCAACTAGGATATCACAGGAGCA-TGTAAATGG-3′, which introduced an *Xba*I and an *Eco*RV site respectively, and amplified the fragment from trkB coding for amino acids Ile37–Thr120. Restriction digestion of Δ2C and this PCR fragment and their subsequent ligation resulted in a plasmid coding for chimera s2B.

For Δ3C the oligonucleotide 5′-GACGTGGCTCACGGAGATCTCA-GGGCTAGCATTGAAAAAGTTCTGCTC-3′ was used to loop-out amino acids Cys133–Leu178 and to introduce an *Nhe*I site and a *Bgl*II site at the 5′- and 3′-ends of the domain respectively. This also inserted an alanine–serine sequence in order to provide a short spacer to the first residue of domain 4, which is a proline. The primers used to amplify trkB domain 3 were 5′-CAGGTCAATCGCTAGCTGTGACATTATGTGG-3′ and 5′-GGTCAACTAGACCGGTTTCGGGTAAAATTGATGTG-3′, which introduced an *Nhe*I and an *Bgl*II site respectively, and amplified the fragment from trkB coding for amino acids Cys123–Leu165. Ligation of this fragment into the cut deletion resulted in a plasmid coding for chimera s3B, carrying an alanine at position 121. This amino acid was mutated back to the original cysteine by site-directed mutagenesis.

For Δ4C the oligonucleotide 5′-GATAGTAGACAGTGAGCGCTA-CGTCACACTGTGAGATGTTCATGCGGAAG-3′ was used to loop-out amino acids Leu178–Ser265 and introduce a *Dra*III site and an *Eco*47III site at the 5′- and 3′-ends of the domain respectively. The primers to amplify trkB domain 4 were 5′-CAGGTCAATCCACAGTGTGAC-TTGCCATCTGCAAATCTG-3′ and 5′-GGTCAACTAGAGCGCTACA-GAATCTTGATCTTCTCC-3′, which introduced a *Dra*III and an *Eco*47III site respectively, and amplified the fragment from trkB coding for amino acids Leu165–Ser247 used for construction of chimera s4B. For Δ4A the oligonucleotide 5′-CGTGTGCAGCTGCACGCTA-GCCGGGAAGGAACCACAGCTAGCATTGGGCATGTG-3′ was used to loop-out amino acids Val160–Val249.

For Δ5C the oligonucleotide 5′-GCGACGGTGGGCACGTGTGAG-ACGCGTTCGTCATGCCCAC-3′ was used to loop-out amino acids Val266–Thr381 and to introduce an *Mlu*I site and a *Pml*I site at the 5′- and 3′-ends of the domain respectively. The primers to amplify trkB domain 5 for construction of s5B were 5′-CAGGTCAATCACGCGT-CTGTCAACCTCACTGTGC-3′ and 5′-GGTCAACTAGCACGTGTG-GGTTTTATCAGTGACGTCTGTG-3′, which introduced a *Mlu*I and a *Pml*I site respectively, and amplified the fragment from trkB coding for amino acids Ser247–Thr395. The primers used to amplify trkA domain 5 for construction of chimera s5A were 5′-CAGGTCAATCAATCA-CGCGTCTGTTCAGGTCAACGTC-3′ and 5′-GGTCAACTAGCACG-TGTGAGTTTTGTCCACCGG-3′, which introduced a *Mlu*I and a *Pml*I site respectively, and amplified the fragment from trkA coding for amino acids Ser244–Val369. For Δ5A the oligonucleotide 5′-GGCACG-GTGGGCACGTGTGAGTACTCCGGACGTTGACCTGAACA-3′ was used to loop-out trkA amino acids Pro252–Val369.

For Δ6C all domains except the second immunoglobulin-like domain were deleted from trkC and the oligonucleotide 5′-GATAGTAGACA-GTGAGCGCTACAGCCAGCACGGAGC-3′ was used to loop-out amino acids Cys1–Val266. For Δ7C all domains except the two immuno-globulin-like domains were deleted from trkC and the oligonucleotide 5′-GTTGACGTGGCTCACGGAGATCTCAGCCAGCACGGAGCC-3′ was used to loop-out amino acids Cys1–Glu180. For Δ6A all domains except the second immunoglobulin-like domain were deleted from trkA and the oligonucleotide 5′-CGTGTGCAGCTGCACGCTAGCAGGG-AAGCTAGCGCCCGCAGATG-3′ was used to loop-out amino acids Pro2–Ser250. For Δ7A all domains except the two immunoglobulin-like domain were deleted from trkA and the oligonucleotide 5′-CCTTCA-GCGTGGGCGCGCTTGCGCTAGCGCCCGCAGATGC-3′ was used to loop-out trkA amino acids Pro2–Val160.

Expression and purification of trk receptors

Plasmid DNA coding for the trk immunoadhesins was introduced into the human fetal kidney cell line 293 by calcium phosphate precipitation (Gorman *et al.*, 1990). The 75% confluent cells were transfected with 20 μg plasmid DNA/15 mm cell culture dish and incubated for 15 h in serum-containing medium. Then the medium was removed and exchanged for serum-free medium (PSO_4) supplemented with 10 mg/l recombinant bovine insulin, 1 mg/l transferrin and trace elements. The supernatant was collected after 48 and 96 h and concentrated to a total volume of 5 ml with Centriprep-30 filtration units (Amicon, Beverly, MA). This concentrated supernatant was applied to a protein A affinity column (1 ml protein A–Sepharose CL-4B; Pharmacia) previously equilibrated with 1.5 M KCl, 50 mM Tris, pH 8.0 (binding buffer). The bound proteins were washed with 10 ml binding buffer and eluted with 2.5 ml 100 mM acetic acid, 150 mM NaCl into 500 μl 1 M Tris, pH 8.0. This solution containing the purified immunoadhesins was concentrated to 1 ml in Centriprep-30 filtration units and 10 ml phosphate-buffered saline (PBS) were added. This solution was again concentrated to 1 ml, sterile filtered and stored at 4°C. Receptor variants did not lose binding activity within 3 weeks of storage under the described conditions.

Iodination of neurotrophins

Purified recombinant human NT-3, BDNF and NGF (Genentech) were labeled by lactoperoxidase treatment using a modification of the Enzymo-bead radioiodination reagent (BioRad) procedure (Escandón *et al.*, 1993). Usually, 2 μg each neurotrophin were iodinated to specific activities ranging from 2000 to 3000 Ci/mmol. The labeled material was stored at 4°C and used within 2 weeks of preparation.

Binding assays

Competition and saturation binding assays were performed using a 96-well plate format and purified receptor and ligand preparations. The binding constants (K_d) of receptor variants are related to inhibition constants (IC_{50}) of competition experiments using labeled and unlabeled neurotrophins by the Cheng–Prusoff equation [$K_d = IC_{50}/(1 + L^*/K_{dL^*})$, where L^* and K_{dL^*} are the concentration of the labeled ligand and its affinity for the receptor respectively; Cheng and Prusoff, 1973]. The concentration of each receptor–immunoadhesin was previously adjusted to bind 10–15% of the total labeled ligand in the absence of competitor in order to satisfy true competition requirements (e.g. $K \times R_t < 0.1$) and to prevent possible artefacts induced by tracer ligand depletion due to increased receptor concentrations (Hulme and Birdsall, 1992). Receptors that did not bind a certain ligand (i.e. trkC and BDNF) were assayed up to a maximal concentration of 200 ng/ml.

After coating each well with 100 μl 5 μg/ml goat $F(ab')_2$ anti-human F_c IgG (Organon Technika, Westchester, PA) in 0.1 M Tris, pH 9.5, for 15 h at 4–8°C, the wells were aspirated, washed three times with PBS, incubated for 2 h with 100 μl of a solution of the receptor–immunoadhesin in binding buffer [Leibovitz's L-15 medium supplemented with 5 mg/ml bovine serum albumin (Intergen, Purchase, PA), 0.1 mg/ml horse heart cytochrome c (Sigma) and 20 mM HEPES, pH 7.2] and washed with PBS. In competition experiments, 50 μl binding buffer was immediately added to the wells in order to prevent drying. Purified, unlabeled neurotrophin was serially diluted in binding buffer to a concentration range of 4096–0.125 pM. Serial dilution was added (25 μl/well) followed by 25 μl labeled neurotrophin. The final concentration of labeled neurotrophin in each well was ~30 pM for trkA, trkB and trkC assays. In saturation binding experiments 75 μl binding buffer was immediately added to the receptor-coated wells followed by 25 μl serial dilutions of labeled ligand. Non-specific binding was measured in the presence of a 100-fold excess of unlabeled ligand (contained in the 75 μl binding buffer) and was determined for each of the receptor variants individually. After 3 h (4 h for saturation assays) incubation at room temperature, the wells were washed with PBS, 0.5% Tween-20 and the bound radioactivity was counted.

Competition data were fitted to a four-parameter equation using the Kaleidagraph software (Abelbeck Software). For saturation experiments, specific binding was calculated as total binding minus non-specific binding. Saturation binding data were analyzed either by fitting bound (pM) versus free (pM) to a single binding site model or by Scatchard transformation and linear regression using the Kaleidagraph software (Abelbeck Software). All saturation and competition binding experiments were repeated at least three times.

Construction of stable transfected NIH 3T3 cells

The cDNA coding for the extracellular domain of the trkC variants Δ5C, Δ6C and s5B, as well as the native human trkB and trkC receptors, were amplified using specific PCR primers which incorporated flanking restriction sites in a manner similar to that described for the construction of the receptor chimeras expressed as immunoadhesins. Similarly, fragments coding for the transmembrane and the intracellular domains of trkC (Δ5c, Δ6c, s5B and trkC) or trkB (trkB) were amplified using PCR. These fragments were ligated into the mammalian expression vector pMEXneo (Martin-Zanca *et al.*, 1989). NIH 3T3 cells were transfected with these constructs using lipofection (DOTAP reagent, Boehringer). At least 10 single colonies for each of the constructs were selected for G418 resistance, expanded and assayed for receptor expression by binding to wheat germ agglutinin or immunoprecipitation with the pan-trk antibody 443 as described (Tsoulfas *et al.*, 1993). For all constructs ≥90% of the selected colonies expressed the desired receptor.

Stimulation of trk receptor autophosphorylation on NIH 3T3 cell lines by neurotrophic factors

Approximately 1×10^7 cells were treated at 37°C for 5 min with a neurotrophin concentration of 100 ng/ml or as indicated in Figure 6. NP-40 plate lysis and immunoprecipitation with antiserum 443 (pan-trk) or 656 (trkC-specific) were performed as previously described (Tsoulfas *et al.*, 1993). The phosphotyrosine content was analyzed by Western blot using monoclonal antibody 4G10 as previously described (Soppet *et al.*, 1991; Tsoulfas *et al.*, 1993). For each of the constructs at least three different cell lines were selected and assayed for neurotrophin-induced autophosphorylation. The results obtained were similar to the ones shown in Figure 5 for all cell lines expressing a particular construct. Stripping of the blots in Figure 5 was done as described (Stephens *et al.*, 1994).

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