Brain-Derived Neurotrophic Factor, Neurotrophin-3, and Neurotrophin-4 Bind to a Single Leucine-Rich Motif of TrkB†

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ABSTRACT: TrkB is a member of the Trk family of neurotrophin receptors. Its extracellular domain exhibits the same modular structure found in its homologs, TrkA and TrkC, consisting of an N-terminal LRM_3 cassette and two immunoglobulin-like modules (Ig2 domain) adjacent to the membrane. The LRM_3 cassette comprises two cysteine-rich clusters framing a tandem array of three leucine-rich motifs (LRMs). On the basis of the recent identification of a nerve growth factor (NGF) binding site within TrkA, the ability of the different structural entities within the extracellular domain of TrkB to bind the various neurotrophins was determined by using a recombinant receptor approach. Brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4 (NT-4) bound to the LRM_3 cassette of TrkB, whereas NGF did not. These binding characteristics evidently reflect *in vivo* specificities. A more precise mapping of the region(s) responsible for binding BDNF, NT-3, and NT-4 identified the second leucine-rich motif of TrkB as a functional unit capable of binding all three neurotrophins. The affinities and kinetics that this short stretch of amino acids exhibited with respect to the different neurotrophins were clearly akin to those observed for cells ectopically expressing TrkB receptors. With 24 amino acids determining the affinities and kinetics of the interactions with three different partners, the leucine-rich motif is strongly established as one of the most potent and flexible protein−protein interaction motifs.

Brain-derived neurotrophic factor (BDNF),[1] neurotrophin-3 (NT-3), and neurotrophin-4 (NT-4) are members of the growing family of neurotrophins (Lindsay et al., 1994; Götz et al., 1994), the paradigm of which is the nerve growth factor (NGF) (Levi-Montalcini, 1987). Neurotrophins are target-derived, dimeric peptide hormones that play an important role in neuronal cell survival and differentiation (Thoenen, 1991; Raffioni et al., 1991; Chao, 1992; Barbacid, 1993). An increasing number of studies has revealed that, among the neurotrophins, BDNF, NT-3, and NT-4, the three ligands of the TrkB receptor tyrosine kinase (Glass et al., 1991; Squinto et al., 1991; Klein et al., 1991b, 1992; Ip et al., 1993; Soppet et al., 1991), have great therapeutic potential in the treatment of neurological disorders and injuries (Lindsay et al., 1994; Schnell et al., 1994; Yan et al., 1992; Koliatsos et al., 1994; Mitsumoto et al., 1992; Ghosh et al., 1994). This fact and the complexity of one receptor interacting with (at least) three different ligands with different pharmacological properties and biological functions (Raffioni et al., 1993) made us interested in the biochemical bases underlying these processes, especially in the primary events in the binding of the three neurotrophins to TrkB.

The cDNA encoding the TrkB tyrosine kinase receptor was first cloned by using a probe specific for the human protooncogene *trk*. The open reading frame encodes a protein approximately 800 amino acids in length, with a calculated M_r for the polypeptide core of 92 000. Studies using antibodies raised against carboxy-terminal sequences of TrkB revealed that the *trk*B gene product is a glycoprotein with an apparent molecular mass of 145 kDa ($p145^{trkB}$), indicating that more than 50% of the extracellular domain is made up of carbohydrates. TrkB was shown to be expressed mainly in the CNS, which gave the first clue that it may serve a function in the development and/or maintenance of the nervous system (Klein et al., 1989; Hofer et al., 1990).

Further studies on *trk*B expression in mouse (Klein et al., 1990) as well as rat (Middlemas et al., 1991) revealed the existence of truncated TrkB receptors ($gp95^{trkB}$) lacking most of the cytoplasmic kinase domain and showing some variation in their C-termini. Rat TrkB has 37% amino acid sequence identity in the extracellular domain and 75% in the kinase domain with the product of the human protooncogene *trk*, the receptor tyrosine kinase TrkA (Middlemas et al., 1991).

Pharmacologically, Trk[2] receptors are characterized by their slow dissociation kinetics, with half-lives of >10 min (Schechter & Bothwell, 1981; Meakin et al., 1992; Meakin & Shooter, 1992; Rodriguez-Tebar & Barde, 1988; Rodriguez-Tebar et al., 1992), which earned TrkA the synonymous designation "slow NGF receptor". It is generally recognized that Trk receptors transduce most of the biologically relevant signals exerted by neurotrophins to the interior

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[1] Abbreviations: CNS, central nervous system; M_r, relative molecular weight; NGF, nerve growth factor; BDNF, brain-derived neurotrophic factor; NT-3, neurotrophin-3; NT-4, neurotrophin-4; LNTR, low-affinity neurotrophin receptor; LRM, leucine-rich motif; LRM_3, LRM_3 cassette; L_2, second leucine-rich motif of TrkB; Ig, immunoglobulin; RT-PCR, reverse transcription−polymerase chain reaction; BSA, bovine serum albumin; MBP, maltose-binding protein; βGal, β-galactosidase; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

[2] In this paper, the receptor family will be referred to as Trk and the individual receptors as TrkA, TrkB, and TrkC.

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of the cell, since the trophic activities of the individual neurotrophins correspond well with the expression of their cognate Trk receptors (Meakin & Shooter, 1992; Lindsay et al., 1994). The importance of TrkB receptors in ontogeny was impressively demonstrated by the targeted disruption of the *trk*B gene in mice, which results in severe nervous system lesions and neonatal death (Klein et al., 1993). Very little is known at this point about the interactions between TrkB and its ligands at the molecular level. BDNF and NT-3 bind to non-neuronal cells ectopically expressing TrkB receptors with affinities in the range of 1 nM (Soppet et al., 1991; Glass et al., 1991; Dechant et al., 1993a). No detailed studies on the interactions between NT-4 and TrkB in terms of binding characteristics are yet available.

There is a second type of neurotrophin receptor, the so-called low-affinity neurotrophin receptor ($p75^{LNTR}$), that is more widely expressed than the Trk receptors and binds all known neurotrophins with similarly low affinities in the nanomolar range, albeit with distinct kinetics (Barbacid, 1993). Pharmacologically, $p75^{LNTR}$ displays characteristics distinctly different from those of the Trk receptors, in that the off-rate is much faster ($t_{1/2} \approx 3$ s) (Meakin et al., 1992). The roles of $p75^{LNTR}$ in the formation of a possible neurotrophin-binding receptor complex and its function in signal transduction are still controversial. Recent studies, however, have indicated that Trk and $p75^{LNTR}$ indeed collaborate in the binding and signal transduction of neurotrophins (Verdi et al., 1994; Barker & Shooter, 1994; Hantzopoulos et al., 1994).

In considering the existence of two neurotrophin receptors that both have, when expressed ectopically, affinities in the low nanomolar range, it is surprising that, on many types of neurons, there seem to be two different types of binding sites for BDNF (Rodriguez-Tebar & Barde, 1988) and NT-3 (Rodriguez-Tebar et al., 1992, 1993; Dechant et al., 1993b; Altar et al., 1993): a small number (a few thousand) of high-affinity receptors ($K_d \approx 10$ pM) and a large number (4−10 times more) of low-affinity sites. There are four hypotheses about how high-affinity binding is achieved: (i) by Trk−Trk homodi/oligomers (Klein et al., 1991a; Jing et al., 1992; Barbacid, 1993), (ii) by Trk−$p75^{LNTR}$ heterodimers (Kaplan et al., 1991; Hempstead et al., 1991), (iii) by a receptor complex of higher order, or (iv) by a yet undiscovered entity.

In this context, it appeared interesting to us to investigate quantitatively which contribution TrkB makes to neurotrophin binding in a possible receptor complex. All of the data published on this issue so far are based on ectopic expression in non-neuronal cells (Soppet et al., 1991; Ip et al., 1993). We chose recombinant receptor proteins for our experiments as the appropriate tool to eliminate any conceivable influences on other receptor components.

TrkB shares with TrkA and a third homologous receptor, TrkC, a unique mosaic of distinct structural modules in the extracellular domain, as identified by Schneider and Schweiger (1991) using special sensitive sequence comparison algorithms. More specifically, an N-terminal LRM_3 cassette and an Ig2 domain are adjacent to the membrane. The LRM_3 cassette consists of two cysteine-rich clusters (containing a total of eight cysteine residues) that are separated by a tandem array of three leucine-rich motifs (LRMs). The Ig2 domain is made up of two immunoglobulin-like modules of the C2 type (Schneider & Schweiger, 1991).

LRM repeats are capable of exerting strong and specific protein−protein interactions. They have been found in proteins as diverse as human glycoprotein Ib (Lopez et al., 1987), *Drosophila* Toll (Keith & Gay, 1990), and *Drosophila* Chaoptin (Krantz & Zipurksy, 1990), wherein they mediate cell−cell interactions and communication, yeast adenylate cyclase, wherein they form the interaction site with the Ras protein (Suzuki et al., 1990), and human ribonuclease (angiogenin) inhibitor, which almost entirely consists of LRMs and exerts one of the strongest protein−protein interactions known so far (Lee & Vallee, 1993; Lee et al., 1988; Schneider et al., 1988). An individual LRM contains some 24 (22−30) amino acids with hydrophobic residues at conserved positions. LRMs are tandemly repeated in a distinct protein, with the number of repeats ranging from a single LRM to as many as 30 (Schneider et al., 1988; Schneider & Schweiger, 1991; Kobe & Deisenhofer, 1994).

What moved TrkB and its ligands BDNF, NT-3, and NT-4 into the focus of great research efforts were a number of studies proving that investigation of the molecular mechanisms by which these molecules interact will be crucial to the understanding and treatment of many neurodegenerative disorders such as Parkinson's and Alzheimer's diseases (Snider, 1994; Klein, 1994; Klein et al., 1993; Ernfors et al., 1994; Mitsumoto et al., 1994). BDNF, NT-3, and NT-4 are also at the center of great hopes in the treatment of injuries to the nervous system, since they have been shown to be capable of preventing adult nerves from degenerating even after the infliction of serious lesions (Schnell et al., 1994; Frisen et al., 1993; Merlio et al., 1993; Yan et al., 1992; Sendtner et al., 1992).

Recently, a distinct biochemical function was assigned to a single LRM for the first time by showing that the isolated second LRM of TrkA is capable of binding nerve growth factor (NGF) (Windisch et al., 1995). This prompted us to investigate whether the LRMs in TrkB also play a role in ligand binding. The fact that a single LRM is sufficient to bind a neurotrophin ligand raised the possibility that the binding sites of BDNF, NT-3, and NT-4 may be assigned to individual LRMs within the LRM_3 cassette of TrkB.

MATERIALS AND METHODS

Neurotrophins. Recombinant *Escherichia coli*/vaccinia virus-expressed mouse BDNF, mouse NT-3 (Götz et al., 1992), and *Xenopus* NT-4 were the kind gifts of R. Kolbeck, G. Dechant, and Y.-A. Barde. Mouse submaxillary gland NGF-β was purchased from Sigma Immuno Chemicals. [^{125}I]-BDNF, [^{125}I]NT-3, and [^{125}I]NT-4 were prepared by using the lactoperoxidase method, and [^{125}I]NGF-β was purchased from Amersham.

Cloning and Expression of Receptor Modules. The regions coding for the different TrkB modules were amplified from rat brain mRNA by RT-PCR and cloned into the pMal^{TM}-p (New England Biolabs) expression vector. The sequences of the fragments were identical to those published in the literature (Middlemas et al., 1991). The construction of the corresponding *trk*A expression vectors is described in Windisch et al. (1995).

The recombinant maltose-binding protein (MBP)−TrkB fusion proteins were expressed in *E. coli*. Twelve different strains were tested for the expression of the highest amounts of soluble protein. The best performance was achieved with

strain M15 (Qiagen), which was transformed beforehand with a second plasmid, pREP4 (Invitrogen). This plasmid carries a kanamycin resistance gene and expresses the lac repressor (lacI) at high levels, which is crucial because the TrkB fusion proteins are highly toxic to *E. coli*. To enhance catabolite repression, all media contained 0.2% glucose. Expression cultures were grown at 29 °C to ODs no higher than 0.5. Freshly transformed cells were used in all cases. Induction was for 90 min at 0.25 mM IPTG. The proteins were purified essentially according to the manufacturer's instructions. Under the preceding conditions, about 10% of the total expressed TrkB fusion proteins were soluble. About 1−3 mg of protein could be purified from a 1 L culture. This number varied somewhat for the individual constructs. The proteins were active without subjecting them to a refolding protocol, as tested by qualitative binding assays (see the following). For the negative controls a fusion protein composed of MBP and β-galactosidase (MBP−βGal) was expressed. The purified proteins were dialyzed extensively against 20 mM Tris Cl (pH 7.4), 200 mM NaCl, and 1 mM EDTA (column buffer) before use in the experiments.

Qualitative Binding Assays. Recombinant protein/assay (500 ng) was batch loaded onto 5 μL of amylose resin in 25 μL of column buffer. After centrifugation, the supernatants were removed and the pellets washed three times with column buffer. In order to minimize nonspecific binding, the column material was pretreated by resuspension in 90 μL column buffer, 5 mg/mL BSA, 0.1 mg/mL cytochrome *c* (bovine heart), 2 mg/mL heat-denatured BSA, and 0.1 mg/mL heat-denatured cytochrome *c* and incubated with gentle shaking for 30 min. [^{125}I]BDNF, [^{125}I]NT-3, [^{125}I]NT-4, or [^{125}I]NGF (5 μL, 4×10^{-9} M) was added and incubated with gentle shaking at 20 °C for 90 min to reach equilibrium binding. Each binding reaction was carried out in duplicate, and for each concentration of [^{125}I]neurotrophin, a MBP−βGal control was made to detect nonspecific binding to MBP. After centrifugation for 3 min, the supernatants were transferred to fresh tubes (SN1). The denatured proteins contained in the buffer very quickly yielded a very solid pellet in this centrifugation step, allowing very fast handling of the samples. The pellets were washed three times in column buffer containing 20 mM maltose to elute the receptor−ligand complexes. The supernatants of all three centrifugation steps were combined in a new tube (SN2). SN1, SN2, and the amylose resin pellet were measured on a gamma counter. SN1 therefore represented free [^{125}I]-neurotrophin, and SN2 represented specifically bound [^{125}I]-neurotrophin. The small amounts of radioactivity trapped in the pellet were added to the free [^{125}I]neurotrophin. Nonspecific binding to the MBP in the control experiments was low (usually <5%) and was subtracted from the specific binding in each case.

Equilibrium Binding Assays. Binding reactions of 100 μL final volume containing 1.25 pmol (100−200 ng, depending on the M_r) of recombinant protein/assay immobilized on 5 μL of amylose resin were prepared as described earlier. Final concentrations of [^{125}I]BDNF and [^{125}I]NT-3 ranged from 7.8125×10^{-12} to 4×10^{-9} M. The samples were processed as described earlier. In addition, experiments with very low concentrations of [^{125}I]neurotrophin were performed with a preincubation time of 270 min to determine whether all binding reactions had actually reached equilibrium binding. All data points are means of duplicates. The data are corrected for nonspecific binding, which did not exceed 10−20% of total binding even at very low concentrations of radioligand.

Kinetics of Association. Binding reactions were prepared as described earlier and contained 2.5 pmol of receptor/100 μL. The starting volume of all reactions was 700 μL. The reactions were brought to final concentrations of [^{125}I]-neurotrophin ranging from 5×10^{-12} to 4×10^{-11} M. Aliquots of 100 μL were taken at the different time points (0, 2, 4, 8, 16, 32, and 64 min) and processed as described earlier. All data points are means of duplicates. The data are corrected for nonspecific binding. Nonspecific binding accounted for maximally 20−25% of total binding at very low concentrations of radioligand and early time points.

Kinetics of Dissociation. Binding reactions of 700 μL were prepared and brought to equilibrium binding as described earlier. Each reaction contained 2.5 pmol of receptor/100 μL. Concentrations of [^{125}I]neurotrophin ranged from 5×10^{-10} to 4×10^{-9} M. Dissociation of [^{125}I]-neurotrophin was induced by the addition of a 100-fold excess of unlabeled neurotrophin. Aliquots of 100 μL were taken at the different time points (0, 2, 5, 15, 30, 60, and 90 min) and processed as described earlier. All data points are means of duplicates.

Inhibition of [^{125}I]BDNF and [^{125}I]NT-3 Binding by NT-4. Binding reactions of 100 μL final volume were prepared as described earlier and contained 2.5 pmol of receptor. The reactions were preequilibrated with concentrations of cold NT-4 ranging from 0 and 2.5×10^{-11} (0.1-fold "excess") to 2.5×10^{-7} M (1000-fold excess) for 60 min. Then the reactions were brought to a final concentration of 2.5×10^{-10} M radioligand ([^{125}I]BDNF or [^{125}I]NT-3), followed by another 180 min incubation. The samples were processed as described earlier. All data points are means of duplicates.

All calculations were performed according to Bylund and Yamamura (1990) and Unnerstall (1990) using the GraFit program (Erithacus).

RESULTS AND DISCUSSION

To identify the contributions of the individual structural entities within the extracellular domain of TrkB to the process of binding each of its three different ligands, brain-derived neurotrophic factor, neurotrophin-3, and neurotrophin-4, these modules were expressed in recombinant soluble form as described in Materials and Methods and tested for their abilities to bind the individual ligands. To do so, the recombinant proteins were immobilized on amylose resin column matrices, and binding of the iodinated neurotrophins to the individual receptor modules was assessed using quantitative binding assays.

By applying a systematic strategy for tracing down the binding site, all three TrkB ligands (BDNF, NT-3, and NT-4) were shown to bind to the extracellular domain of TrkB, whereas NGF showed no detectable affinity (Table 1). Conversely, NGF bound to the corresponding domain of TrkA as described previously (Windisch et al., 1995), while BDNF, NT-3, and NT-4 did not. These results clearly demonstrated *in vivo* specificities for these recombinant proteins (Raffioni et al., 1993; Meakin & Shooter, 1992; Lindsay et al., 1994; Barbacid, 1993).

There has, however, been some controversy over the possible activation of TrkA by NT-3 and NT-4. Work from

Table 1: Mapping of a Universal Neurotrophin Binding Site in Trk Tyrosine Kinase Receptors[a]

		[^{125}I]neurotrophin			
	module	BDNF	NT-3	NT-4	NGF
TrkB	Ex	0.93 ± 0.17	1.32 ± 0.33	+	−
	LRM_3	1.16 ± 0.23	1.43 ± 0.29	+	−
	Ig2	−	−	−	−
	L_{1-3}	+	+	+	−
	L_2	1.39 ± 0.21	1.17 ± 0.43	+	−
TrkA	Ex	−	−	−	1.29 ± 0.20

[a] A binding site for BDNF, NT-3, and NT-4 could be tracked down to a single LRM within the extracellular domain of rat TrkB. The equilibrium binding assays were performed as described in Materials and Methods. All K_d values are in 10^{-9} M ± standard deviation. The abbreviations in this table are as follows. TrkB: Ex, entire extracellular domain (C21−E417); LRM_3, LRM_3 cassette (C21−P186); Ig2, Ig2 domain (S187−E417); L_{1-3}, LRM_3 cassette lacking both cysteine clusters (L61−L132); L_2, isolated second LRM (T86−I109); the residue numbers here are stated according to Middlemas et al. (1991). TrkA: Ex, C36−E416 [according to Meakin et al. (1992)]. Symbols: +, neurotrophin binding; −, no neurotrophin binding detectable.

several groups has indicated that NT-3 (Cordon-Cardo et al., 1991; Rosenthal et al., 1990) and NT-4/5 (Berkemeier et al., 1991) are capable of eliciting tyrosine autophosphorylation of TrkA and biological responses in cells expressing this receptor, but lacking TrkB. Other investigations performed on different cellular systems showed no such responses in cells expressing TrkA receptors, even when treated with high concentrations of NT-3 or NT-4 (Ernfors et al., 1990; Squinto et al., 1991; Ip et al., 1993). In terms of the biochemistry of the interaction of these ligands with TrkB, the results presented here can contribute to unraveling these controversies. NT-3 and NT-4 did not exhibit any detectable affinity for TrkA in our assays, which means (taking into consideration the sensitivity of such an assay) that their actual affinity *in vivo* is at least 3 orders of magnitude lower for TrkA ($K_d > 10^{-6}$ M) than for TrkB. This explains why activation of TrkA, if at all, is only seen at extremely high concentrations of NT-3 or NT-4. The results presented here therefore represent the first clear account of the interactions of four different neurotrophins with their receptors TrkA and TrkB, independent from the imponderabilities arising from cell-type-specific differences.

Another interesting detail of these studies using *E. coli*-expressed recombinant receptor proteins was the fact that glycosylation does not seem to play a major role in the interaction of BDNF, NT-3, and NT-4 with TrkB, thus behaving the same way as NGF with respect to TrkA. This is intriguing considering that more than 50% of the molecular mass of the extracellular domain of TrkB *in vivo* is made up of carbohydrate moieties. It has been previously shown for the interaction of several ligands with their receptors that glycosylation is not necessarily an important determinant of these processes (Schwarz et al., 1991; Sairam, 1989), whereas in other cases (Frost et al., 1991; Moller et al., 1993) it seems to contribute considerably to ligand-binding affinity and/or specificity.

Further narrowing down of the binding site(s) for BDNF, NT-3, and NT-4 in TrkB was accomplished by expressing the two major structural components of its extracellular domain, the LRM_3 cassette and the Ig2 domain. The binding assays rendered the LRM_3 cassette capable of binding all three neurotrophins, whereas in this system the Ig2 domain bound none. This suggests that the same respective structural unit is capable of ligand binding in all Trk-type receptors (Windisch et al., 1995).

As mentioned earlier, domains containing repeats of leucine-rich motifs have been implicated in tight and highly specific protein−protein interactions (Schneider et al., 1988; Lee & Vallee, 1993; Lopez et al., 1987; Keith & Gay, 1990; Krantz & Zipursky, 1990; Suzuki et al., 1990). It therefore is not surprising that the specificity observed in our experiments (Table 1) could be exerted by such a domain. More surprising was the apparent lack of binding affinity of the Ig2 domain in our experiments. Immunoglubulin-like domains have been shown to be potent ligand-binding entities in the keratinocyte growth factor receptor (Yayon et al., 1992), the macrophage colony-stimulating factor receptor (Wang et al., 1993), and intercellular adhesion molecule 1 (ICAM-1) (Diamond et al., 1991). In the case of Trk receptors, however, the immunoglobulin-like domains seem to play a role different from primary ligand binding, although they certainly have great importance in specifically mediating biologically relevant neurotrophin signals to the inside of the cells. It has been shown that a mutation in the second immunoglobulin-like domain of TrkB (C345S) abolishes the ability of NT-4 to morphologically transform 3T3 fibroblasts expressing TrkB, whereas it has no effect on the activity of BDNF, the second primary TrkB ligand (Ip et al., 1993). This indicates that the LRM_3 cassette may provide initial binding affinity and specificity, allowing the Ig2 domain to interact with certain regions of the neurotrophins and conveying the signal to the inside of the cell. Binding of the neurotrophin to the LRM_3 cassette may also lead to a conformational change within the receptor not observed in our system, thus "handing over" the neurotrophins to the Ig2 domain. Considering that only one in ten receptors may undergo such a conformational change, this step could be crucial in the creation of high-affinity binding to Trk receptors, thus leading to an active receptor complex. Since it is very possible for such an event to only take place between individual protomers within a receptor dimer to be formed, this proposed mechanism would also explain how high-affinity receptors are created by the formation of Trk receptor homodimers (Barbacid, 1993; Meakin & Shooter, 1992; Jing et al., 1992).

More detailed analysis of the structures within the LRM_3 cassette responsible for the binding of the individual neurotrophins yielded surprising results (Table 1). Regarding the presence of three LRM repeats within each of the Trk-type receptors, it seemed like a plausible hypothesis that the binding of the various neurotrophins might be assigned to distinct LRM repeats within the LRM_3 cassette. However, the results presented here indicate that a 24-amino acid, single leucine-rich motif of TrkB, namely, the second one (L_2), binds all three ligands, BDNF, NT-3, and NT-4.

We have shown previously that the corresponding LRM of TrkA is responsible for binding this receptor's primary ligand, NGF. The second LRM of rat TrkB shares 13 identical amino acids with the second LRM of rat TrkA, leaving 11 amino acid residues to account for the specificity of binding 3 different neurotrophins. This firmly establishes the leucine-rich motif (LRM) as one of the most potent and flexible protein−protein interaction motifs.

To find out quantitatively what contribution such a minimal clearly defined binding site can make to the binding

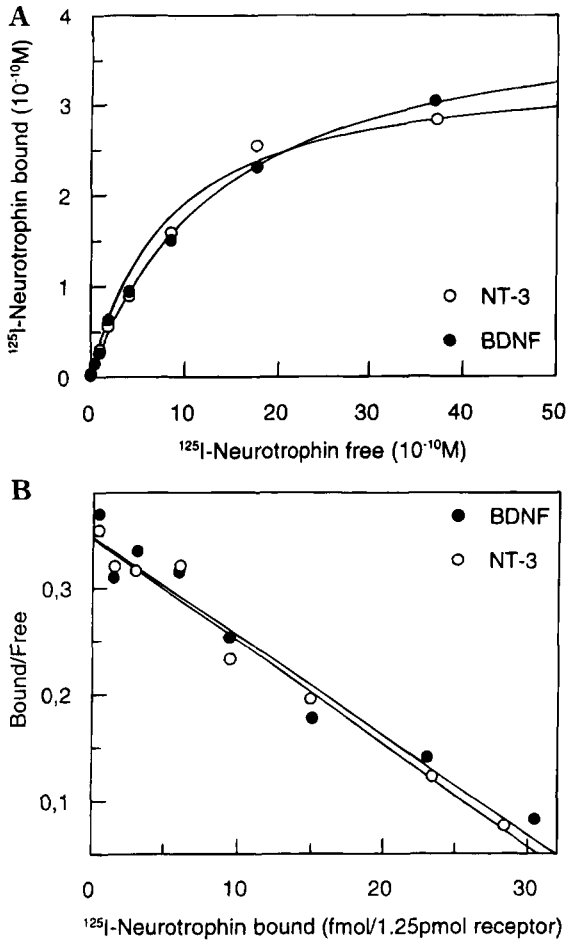

FIGURE 1: (A) Binding of [^{125}I]BDNF and [^{125}I]NT-3 to the 24-amino acid second LRM of TrkB. The experiments were performed as described in Materials and Methods using immobilized recombinant receptor proteins. The stoichiometry of neurotrophin binding was in a similar range for all receptor proteins tested. Final concentrations of [^{125}I]BDNF or [^{125}I]NT-3 ranged from 7.8125 × 10^{-12} to 4 × 10^{-9} M. (B) Scatchard plot analysis of the equilibrium binding data. [^{125}I]BDNF and [^{125}I]NT-3 bind to TrkB L_2 with K_d's of 1.39 and 1.17 nM, respectively. No binding sites in the range 1 × 10^{-11} M could be detected in these assays.

affinity of the receptor complex *in vivo*, the equilibrium binding constants (K_d's) of the interactions of [^{125}I]BDNF, [^{125}I]NT-3, and [^{125}I]NT-4 with the second LRM (L_2), as well as with the LRM_3 cassette and the entire extracellular domain (Ex) or TrkB, were determined (Table 1). Surprisingly, the differences in the affinities of Ex, LRM_3, and L_2 for each of the ligands were minimal. This indicates that we have indeed mapped the only binding site present in the receptor in this conformational stage. The affinities of L_2 for BDNF and NT-3 are very similar, with K_d's of approximately 1 nM as determined by steady state binding (Table 1, Figure 1A,B). These values are in good agreement with those obtained in experiments with cells ectopically expressing TrkB receptors. Soppet et al. (1991) found that mouse NIH 3T3 fibroblasts expressing rat TrkB (which is what was used in this study) bind [^{125}I]BDNF with a K_d of ~1.8 × 10^{-9} M and [^{125}I]NT-3 wtih a K_d of ~1.3 × 10^{-9} M. Dechant et al. (1993a) determined a very similar value for [^{125}I]BDNF binding to chick TrkB ectopically expressed in the human embryonic kidney cell line A293. This indicates that L_2 represents a binding site that is of quantitative importance within the TrkB receptor. However, it does not display the high-affinity-binding component exhibited by embryonic chick dorsal root ganglia (Rodriquez-Tebar & Barde, 1988; Rodriguez-Tebar et al., 1992), sympathetic (Rodriguez-Tebar et al., 1993), or other neurons. In these studies, the authors were able to detect high-affinity binding [K_d's of ~1.7 × 10^{-11} M for both [^{125}I]BDNF and [^{125}I]NT-3) in equilibrium binding experiments using concentrations of radioligand around or below 1.8 × 10^{-11} M. Even at [^{125}I]neurotrophin concentrations as low as 7 × 10^{-12} M, with nonspecific binding not rising above 10−20%, we were unable to detect such high affinities. One possible explanation for why we might not have been able to detect high-affinity binding in our assays is that in the proper cellular environment the neurotrophin could be transferred from L_2 to a high-affinity binding site within the Trk receptor formed by a conformational change upon ligand binding (see above). High-affinity binding may also require the action of additional receptor components [membrane proteins or extracellular matrix components such as proteoglycans, as has recently been shown for NT-6 (Götz et al., 1994)], which might only be present (or present in the correct stoichiometric relations) in such neurons. Other researchers have suggested that intact signal transduction pathways may be required for the generation of high-affinity binding sites (Raffioni et al., 1993). $p75^{LNTR}$ has been discussed as a possible factor that could turn on these pathways, possibly leading to some posttranslational modification of Trk receptors or to association with other receptor components (Meakin & Shooter, 1992).

NT-4 did not yield consistent results in our equilibrium binding assays, in that the data points obtained with different concentrations of radioligand could not be combined into a reasonable saturation curve. This may be in part due to the fact that the affinity of NT-4 for TrkB L_2 is at the edge of detectability in our system, with the percentage of nonspecific binding in total binding becoming unduly high. Alternatively, there may be complex mechanisms involved in the association and/or dissociation of NT-4 to/from L_2, thus influencing the kinetics and the measurability of K_d. For this reason, we resorted to inhibition experiments competing [^{125}I]NT-4 binding to L_2 with different concentrations of BDNF and NT-3 (Figure 2). In these experiments, the IC_{50} of the NT-4 inhibition curve at 2.5 × 10^{-10} M [^{125}I]BDNF was at 5.90 × 10^{-10} M, which corresponds to a K_i of 4.99 × 10^{-10} M. The IC_{50} value of NT-4 with respect to [^{125}I]-NT-3 was at 6.35 × 10^{-10} M, yielding a K_i of 5.23 × 10^{-10} M. This clearly shows that NT-4 can also bind to TrkB via the second LRM with practically as high an affinity as BDNF and NT-3. This corresponds well with results that have identified NT-4 as a fair ligand of TrkB as far as binding affinity is concerned (Dechant et al., 1993a; Klein et al., 1992) and even as a "preferential" ligand as far as the biological NT-4 effects mediated by this receptor are concerned (Ip et al., 1992, 1993).

To analyze in more detail the molecular mechanism by which L_2 binds its ligands, we also investigated the kinetics of association and dissociation with BDNF and NT-3. It has been shown that kinetic analyses are a more sensitive approach to detect possible high-affinity binding sites for neurotrophins than are steady state binding experiments (Rodriguez-Tebar & Barde, 1988). On the basis of steady state binding data, we used higher concentrations of [^{125}I]-neurotrophins in our initial experiments. The resulting data were difficult to interpret, but implied that at least part of

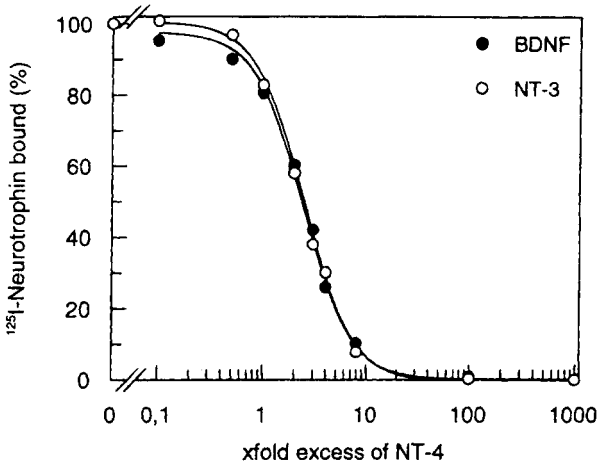

FIGURE 2: Inhibition of NT-4 of the binding of [^{125}I]BDNF and [^{125}I]NT-3 to TrkB L_2. NT-4 binds to the same binding site, the second LRM, as BDNF and NT-3 within the extracellular domain of TrkB. The recombinant receptor proteins were preincubated with various concentrations of NT-4 representing a 0/0.1- to 1000-fold excess over the subsequently added iodinated neurotrophin. Samples were treated as described in Materials and Methods. The K_i values of NT-4 were 4.99×10^{-10} M with respect to [^{125}I]BDNF and 5.23×10^{-10} M with respect to [^{125}I]NT-3.

the binding occurred with on-rates faster than the $\sim 3 \times 10^5$ M^{-1} s^{-1} expected for a K_d of 1 nM from the literature (Rodriguez-Tebar & Barde, 1988). Therefore, [^{125}I]neurotrophin concentrations ranging from 5×10^{-12} to 4×10^{-11} M were used in the following association experiments, which is a concentration range well fit to detect fast on-rates. Even though the standard two-step sucrose gradient centrifugation method proved incompatible with our amylose resin system, the inclusion of denatured proteins in our assays allowed the speedy handling of samples required to detect such rapid binding processes and obtain interpretable data. The association kinetics (Figure 3) of both [^{125}I]BDNF and [^{125}I]-NT-3 to L_2 at these concentrations of radioligand seem to be diffusion controlled ($k_{+1} = 2.70 \times 10^7$ and 3.30×10^7 M^{-1} s^{-1}, respectively), which corresponds to the kinetics of high-affinity BDNF and NT-3 receptors (Rodriguez-Tebar & Barde, 1988). This is a result that is difficult to interpret since it implies that there are high-affinity binding sites present in our system that we were unable to detect in our steady state binding experiments. It could also mean that association of neurotrophins to Trk receptors follows a more complex mechanism than is the case in most receptor–ligand interactions.

The dissociation kinetics for both neurotrophins, with half-lives of about 41.5 min for [^{125}I]BDNF and 19.5 min for [^{125}I]NT-3 (Figure 4A,B), are in good agreement with previously performed experiments in the cellular system (Rodriguez-Tebar & Barde, 1988; Rodriguez-Tebar et al., 1992, 1993). As in the measurements using [^{125}I]BDNF and dorsal root ganglia (DRG) neurons (Rodriguez-Tebar & Barde, 1988), no biphasic behavior [as observed by Rodriguez-Tebar et al. (1992) for the dissociation of [^{125}I]NT-3 from sensory neurons] could be detected in the dissociation of either [^{125}I]BDNF or [^{125}I]NT-3 from any of the recombinant TrkB receptor modules. The dissociation behavior of the labeled neurotrophins in the presence of an excess unlabeled ligand was determined after preincubation with several different concentrations of radioligand, leading to different levels of receptor occupancy. In these experiments

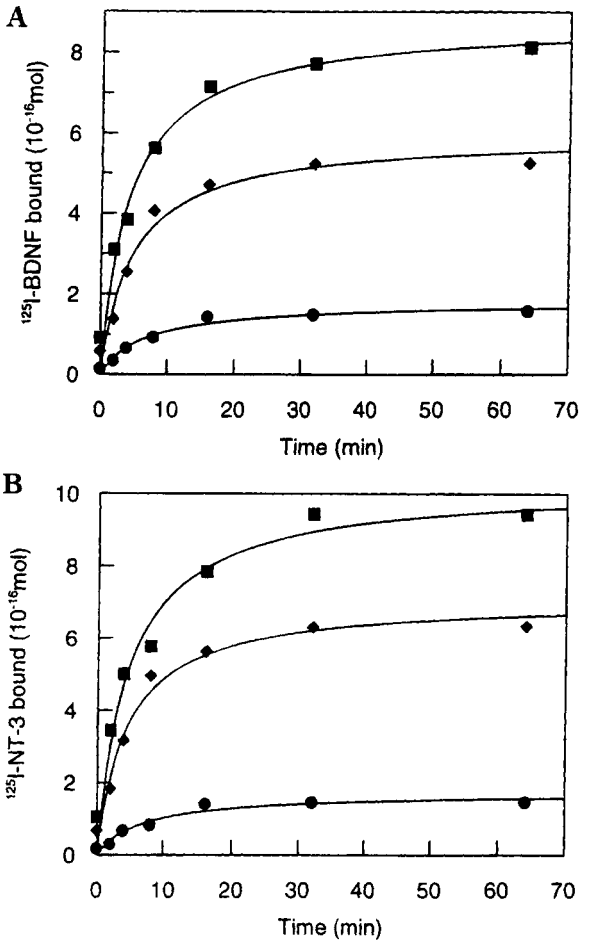

FIGURE 3: (A) Kinetics of association of [^{125}I]BDNF to TrkB L_2. The experiments were performed as described in Materials and Methods. Concentrations of [^{125}I]neurotrophin were chosen in the low range (5×10^{-12} to 4×10^{-11} M) to detect binding sites with high on-rates. The data are corrected for nonspecific binding. The k_{+1} value for the association of [^{125}I]BDNF with TrkB L_2 is 2.70×10^7 M^{-1} s^{-1}. Symbols: ●, 5 pM; ◆, 20 pM; ■, 40 pM [^{125}I]-neurotrophin. (B) Kinetics of association of [^{125}I]NT-3 with TrkB L_2. The experiments were performed as described earlier. The k_{+1} value for the association of [^{125}I]NT-3 with TrkB L_2 is 3.30×10^7 M^{-1} s^{-1}. The symbols are the same as in (A).

the dissociation behavior depended on receptor occupancy, implying a simple mechanism of dissociation.

To corroborate the results of the kinetic binding studies, additional steady state binding experiments were performed to exclude the possibility that some of the observed kinetic phenomena were due to nonequilibrium effects. An increase in the preincubation time in steady state experiments by 3-fold to 270 min did not lead to the appearance of an additional component in the Scatchard analysis. Taken together, the kinetics of association and dissociation demonstrate an astonishingly high degree of complexity exerted by as few as 24 amino acids, which appear to be capable of exhibiting differentiative characteristics for distinct neurotrophins.

The complex relationships concerning the interactions of NGF, BDNF, NT-3, and NT-4 with TrkA and TrkB are summarized in Figure 5. A single leucine-rich motif of TrkB appears to be capable of mediating the binding of three different neurotrophins, BDNF, NT-3, and NT-4, with distinct affinities and kinetics. Taking into consideration that 13 amino acids are identical between L_2 of rat TrkB and L_2

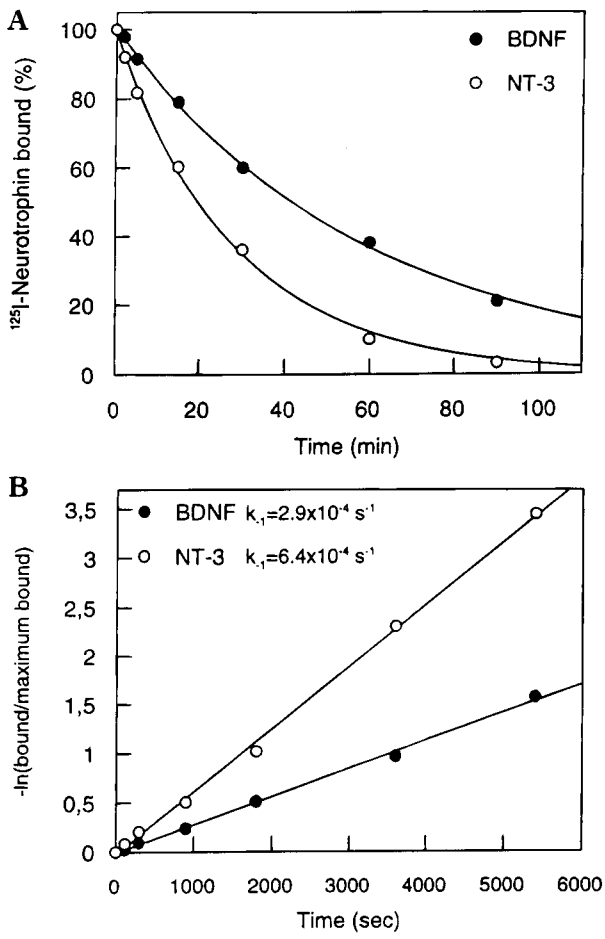

FIGURE 4: (A) Kinetics of dissociation of [^{125}I]BDNF and [^{125}I]-NT-3 from TrkB L_2. The experiments were performed as described in Materials and Methods. Concentrations of [^{125}I]neurotrophin ranged from 5×10^{-10} to 4×10^{-9} M. Dissociation of [^{125}I]neurotrophin was induced by the addition of a 100-fold excess of unlabeled neurotrophin. Aliquots were taken at the different time points and processed as described earlier. (B) Semilogarithmic plot of the dissociation data. The $t_{1/2}$ and k_{-1} values for [^{125}I]-BDNF↔TrkB L_2 were 41.5 min and 2.9×10^{-4} s^{-1}, respectively. The corresponding values for NT-3 were 19.5 min and 6.4×10^{-4} s^{-1}.

of rat TrkA, which has been shown to exclusively bind NGF, only 11 residues are left to account for the binding specificity, affinity, and kinetics of the interaction with three different neurotrophins. *In vivo* these three neurotrophins exert clearly distinguishable biological functions through one and the same receptor, TrkB (Ip et al., 1992, 1993; Ibanez et al., 1993; Klein et al., 1992; Meakin & Shooter, 1992; Raffioni et al., 1993). Considering the distinct biochemical/pharmacological properties BDNF, NT-3, and NT-4 exhibit toward L_2, it must be assumed that this region of TrkB substantially contributes to the discrimination of these neurotrophins *in vivo*, even though we presume the involvement of other parts of the receptor, e.g., the immunoglobulin domains (Klein et al., 1992; Ip et al., 1993), in this process.

The elucidation of the molecular mechanisms by which proteins containing LRM repeats interact with their respective partners is not merely of intellectual interest. The information about the interactions between LRM−proteins and their ligands, the recently determined 3D structures of an LRM−protein in complex with its ligand (Kobe & Deisenhofer, 1995), as well as of the two LRM−ligands NGF (McDonald et al., 1991) and NT-4 (Fandl et al., 1994; Ip et al., 1992),

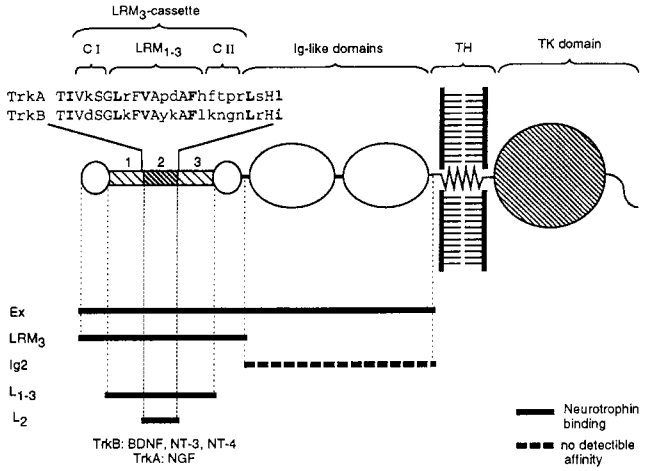

FIGURE 5: Structure of Trk receptors and neurotrophin binding. BDNF, NT-3, and NT-4 bind to the second LRM repeat of TrkB, and NGF binds to the corresponding repeat of TrkA (Windisch et al., 1995). Both sequences contain six hydrophobic residues at conserved positions and share 13 identical residues. The specificity of the complex interactions of four distinct ligands with two different receptors therefore is determined by a mere 11 amino acid residues.

and the data that have been obtained from mutagenesis studies of the neurotrophins (Ibanez et al., 1993; Suter et al., 1992; Urfer et al.,1994; Drinkwater et al., 1993; Kahle et al., 1992) defining the regions responsible for binding to Trk receptors clearly will be of value in the construction of "custom-made" neurotrophins designed for the support of specific neuronal populations.

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