Specific neurotrophin binding to leucine-rich motif peptides of TrkA and TrkB

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Abstract The extracellular domains of the TrkA and TrkB neurotrophin receptors contain defined structural modules such as immunoglobulin-like domains and leucine-rich motifs (LRMs) [Schneider and Schweiger, Oncogene 6 (1991) 1807–1811]. Recently, the second LRM of TrkA was identified as a functional nerve growth factor (NGF) binding site [Windisch et al, J. Biol. Chem. (1995) in press]. A peptide corresponding to this region effectively bound NGF and blocked binding of NGF to the recombinant extracellular domain of TrkA. The corresponding TrkB peptide exhibited the same effects with respect to brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4 (NT-4), indicating that all three TrkB ligands utilize this same binding site. Isolated LRMs therefore embody independent functional entities.

Key words: Affinity chromatography; Binding site; Leucine-rich motif; Nerve growth factor; Peptide blocking; Tyrosine kinase receptor

1. Introduction

TrkA is the biologically functional receptor for nerve growth factor (NGF) [9,13], whereas its homolog TrkB binds three different neurotrophins [3,8,16,24,36] namely brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and neurotrophin-4 (NT-4) [5,14,15,31,32]. The extracellular domains of Trk-type receptors harbor a modular mosaic of potential ligand binding modules, namely two immunoglobulin (Ig)-like domains and an LRM_3-cassette consisting of a tandem array of three leucine-rich motifs (LRMs) flanked by cysteine-rich clusters [28]. These structures were discovered by the application of sequence comparison algorithms capable of detecting even highly degenerate motifs [30].

LRMs are short amino acid sequences of 22 to 30 residues that are tandemly repeated in an individual protein and contain hydrophobic residues at conserved positions [27,29]. Repeats of LRMs have been known as potent mediators of strong and specific homo- or heterophilic protein–protein interactions. They have been found in proteins as diverse as human platelet glycoprotein IX [7], *Drosophila* Toll [11] and *Drosophila* Chaoptin [18], where they mediate cell–cell interactions and communication, and yeast adenylate cyclase, where they form the interaction site with the Ras protein [35]. However, only recently the first single LRM with a defined function was identified. An NGF binding site was mapped to the second LRM of TrkA using a recombinant receptor approach [39].

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Based on this information we synthesized a 24 amino acid peptide corresponding to this region of TrkA (L2A) to investigate whether this module represents an independent functional and structural unit in that it can bind NGF and, by doing so, inhibit NGF binding to the recombinant extracellular domain of TrkA. To investigate whether this 24 amino acid LRM is the smallest possible functional unit capable of mediating NGF binding and if a different phasing of the LRM repeats as suggested by Kobe and Deisenhofer [17] leads to the same results, a peptide with the same sequence as L2A but lacking three amino acids at the N-terminus as well as a recombinant fusion-protein shifted five residues toward the N-terminus with respect to L2A were produced and tested for their ability to block NGF binding to TrkA.

Given the information about the NGF binding site within the TrkA receptor, it was obvious to extend the application of this peptide approach to TrkB. TrkA and TrkB share 37% amino acid sequence identity in the extracellular domains and 75% in the kinase domains [23,28]. Despite this high degree of similarity, TrkB is a much more versatile receptor than TrkA in that it binds three different ligands [24] with clearly distinguishable pharmacological characteristics [2,22,25,26] leading to distinct biological effects [8,14,15]. In order to show whether the second LRM also plays a role in ligand binding in the TrkB receptor, a peptide corresponding to this region (L2B) was synthesized and characterized in the same way as L2A. Investigating the inhibitory effect of different concentrations of L2B on the binding of BDNF, NT-3 and NT-4 to the recombinant extracellular domain of TrkB should give precise information in qualitative and quantitative terms on the role of this region in the binding of the different ligands.

2. Experimental

2.1. Neurotrophins

Recombinant *E. coli*/vaccinia virus expressed mouse BDNF, mouse NT-3 [6], and *Xenopus* NT-4 were 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 using the lactoperoxidase method, [^{125}I]NGF-β was purchased from Amersham.

2.2. Peptides

Peptides corresponding to the second leucine-rich motif of rat TrkA and rat TrkB were chemically synthesized and purified. The phasing of the leucine-rich motif peptides used in this work was chosen in accordance with the data obtained in previous studies [38]. The amino acid sequences were derived from the published cDNA sequences [22,23]. Both peptides were 24mers with the sequences NH_2-TIVKSGLRFVAPDAFHFTPRLSHL-COOH for TrkA (peptide 'L2A') and NH_2-TIVDSGLKFVAYKAFLKNGNLRHI-COOH for TrkB (peptide 'L2B'), respectively. The sequence of the control peptide L2A(−3) is identical to that of L2A but lacks the first three amino acids (NH_2-KSGLRFVAPDAFHFTPRLSHL-COOH).

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2.3. Construction of peptide affinity columns

The L2A and the L2B peptides were covalently coupled via amide bonds to *N*-hydroxysuccinimide ester-activated cross-linked agarose gel beads (Affi-Gel 10, Bio-Rad) essentially according to the manufacturer's instructions. 10 mg of the respective peptide were incubated with 2 ml of precycled column material in 0.1 M MOPS, pH 7.0 with gentle agitation over night. Remaining active ester groups were blocked by the addition 0.2 ml 1 M ethanolamine pH 8.0 followed by a further incubation period of 2 h. The column materials were washed with at least ten volumes of phosphate buffered saline (PBS) containing 1 M NaCl and 1 mg/ml bovine serum albumin (BSA) before using them in the experiments.

2.4. Binding of [^{125}I]NGF and [^{125}I]BDNF to the peptide affinity columns

Two times 0.5 ml of the L2A and the L2B affinity column materials were packed into small columns and washed with ten column volumes PBS containing 1 mg/ml BSA and 0.1 mg/ml cytochrome *c* (bovine heart) to reduce non-specific binding. 0.2 ng (~23000 cpm) of [^{125}I]NGF in 200 μl of the above buffer were applied to an L2A column. Starting at this point, fractions of 0.5 ml were taken. The column was washed with six volumes of buffer before adding 5 mg of the L2B peptide in 0.5 ml of buffer (first elution step). Again the column was washed with three volumes of buffer. Then 5 mg of the L2A peptide in 0.5 ml of buffer were added (second elution step) followed by another wash of five column volumes. All fractions were measured in a gamma counter.

Three additional analogous experiments were performed. [^{125}I]NGF was applied to an L2B column followed by elution steps with soluble L2B and L2A. [^{125}I]BDNF was also applied to an L2B column followed by elution steps with soluble L2A and L2B. Finally, [^{125}I]BDNF was applied to an L2A column followed by elution steps with soluble L2A and L2B. Again, all obtained fractions were measured in a gamma counter.

2.5. Production and purification of recombinant receptor proteins

The regions encoding the extracellular domains of rat TrkA (C36-E416) and rat TrkB (C21-E417) as well as of the region encoding L2A' (E92-N115 of rat TrkA) were amplified from embryonal rat brain mRNA by RT-PCR and cloned into the pMal™-p expression vector. The sequences of the fragments were identical to the ones published in the literature [22,23]. The recombinant Maltose Binding Protein (MBP)-TrkA/B fusion proteins were expressed in *E. coli* and purified by amylose resin affinity chromatography essentially as described previously [38,39]. For the negative controls a fusion protein composed of MBP and β-galactosidase (MBP-βGal) was expressed. The purified proteins were extensively dialysed against 20 mM Tris-HCl pH 7.4, 200 mM NaCl, 1 mM EDTA (column buffer) before using them in the experiments. The production of the $L_{1.5\text{-}3}C_2$ and the $C_1L_{1\text{-}1.5}$ proteins is described in [39].

2.6. Binding inhibition assays

2.6.1. Immobilization of recombinant receptors. 100 ng (2.5 pmol) of recombinant protein/assay were batch loaded onto 5 μl of amylose resin in 25 μl of column buffer. After centrifuging, the supernatant was removed and the pellet washed three times with column buffer. In order to minimize non-specific binding, the column material was resuspended in 85 μl column buffer + 5 mg/ml BSA + 0.1 mg/ml cytochrome *c* (bovine heart) + 2 mg/ml heat denatured BSA + 0.1 mg/ml heat-denatured cytochrome *c* and incubated with gentle shaking for 30 min. The denatured proteins were included to give a more solid amylose resin pellet after centrifugation.

2.6.2. Inhibition assays. [^{125}I]NGF or [^{125}I]BDNF were incubated with gentle shaking at 20°C for 90 min with different amounts of L2A, L2B or L2A(−3) in a total volume of 10 μl. The buffer used was column buffer containing 5 mg/ml BSA and 0.1 mg/ml cytochrome *c*. After this period, 90 μl of the corresponding immobilized recombinant receptor were added followed by another 90 min incubation to reach equilibrium binding. The final concentrations of the peptides ranged from 0 M, 1.86×10^{-8} M to 3.72×10^{-5} M. The final concentration of both [^{125}I]NGF and [^{125}I]BDNF was 1×10^{-9} M in all experiments. After centrifuging for 3 min, the supernatants were transferred to fresh tubes (SN1). 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 'Cobra Auto Gamma' counter (Packard). SN1 therefore represents free [^{125}I]neurotrophin, SN2 specifically bound [^{125}I]neurotrophin. The small amounts of radioactivity trapped in the pellet were added to the free [^{125}I]neurotrophin. For each concentration of [^{125}I]NGF/[^{125}I]BDNF a MBP-βGal control was included to detect non-specific binding to MBP. In general non-specific binding was very low. It was subtracted from the value for specific binding in each case. All binding reactions were carried out in duplicate. Differences between duplicate values were generally small. K_i values were calculated according to [1].

3. Results and discussion

To determine whether the second leucine-rich motif of TrkA can form the proper structures and function in the absence of its natural molecular context or of a supporting carrier protein [39], the ability of a peptide corresponding to this functional module (amino acids 97 to 120 of rat TrkA according to Meakin et al. [22]) was synthesized (L2A). The peptide was covalently coupled to an agarose column matrix as described in section 2. If the L2A peptide represents a functional entity, the resulting column material should be suitable for the application as a highly specific neurotrophin affinity chromatography matrix. To test this hypothesis, a respective column was loaded with radioactively labelled (iodinated) NGF ([^{125}I]NGF). The columns were washed with several volumes of buffer but only about 70% of the radioactivity loaded onto the column reappeared in the flow through fractions (Fig. 1). This was a strong indication that [^{125}I]NGF had actually bound to the immobilized L2A. This again indicates that this isolated leucine-rich motif, even when covalently attached to a column matrix, represents a functional unit in that it forms a structure that positions its side chains at the adequate 3D coordinates to properly interact with the crucial residues of the ligand. It is therefore one of the smallest self-contained growth factor binding sites known at this point.

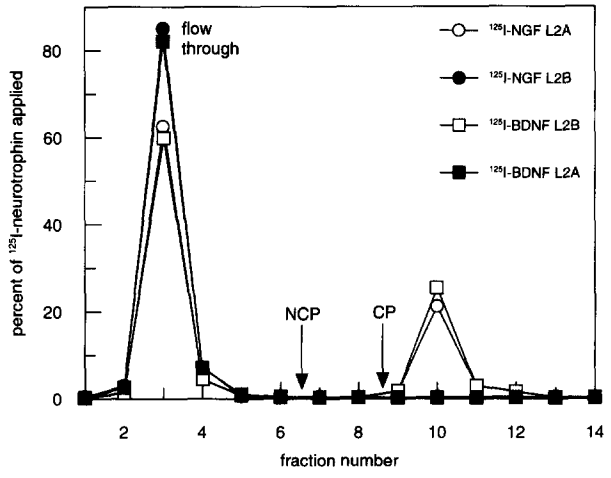

Fig. 1. Binding of [^{125}I]NGF to a TrkA L2A affinity column and of [^{125}I]BDNF to a TrkB L2B affinity column. The experiments were performed as described in section 2. [^{125}I]NGF or [^{125}I]BDNF were applied to L2A and L2B affinity columns, the columns washed and the [^{125}I]neurotrophins eluted in two steps by the addition of the Trk peptides. NCP (non-competitive peptide) and CP (competitive peptide) indicate the addition of the L2A or L2B peptides. In experiments involving [^{125}I]NGF NCP=L2B and CP=L2A, in those involving [^{125}I]BDNF NCP=L2A and CP=L2B, so that in each case the peptide derived from the non-natural receptor was added first.

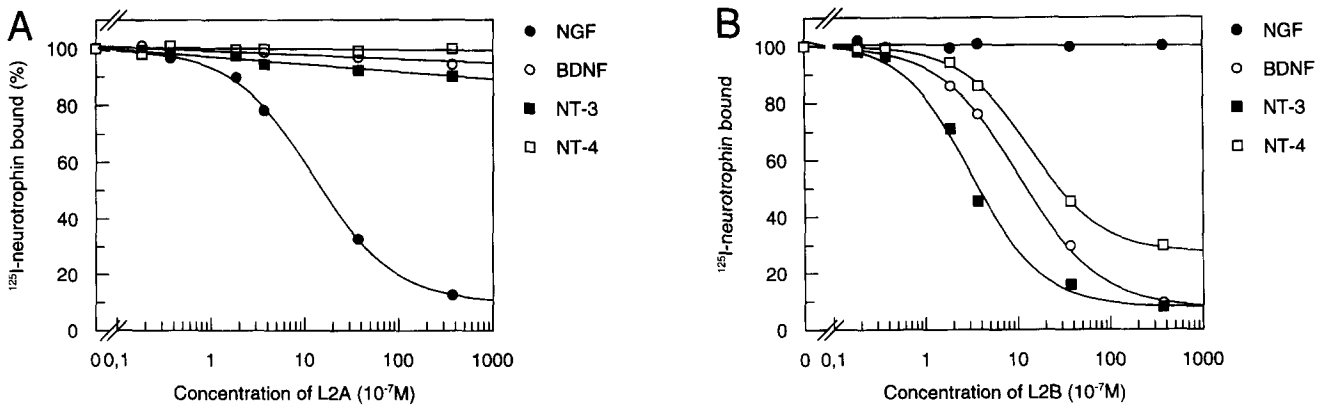

Fig. 2. Effective competition by L2A/L2B of [^{125}I]neurotrophin binding to the TrkA/TrkB recombinant extracellular domains. The experiments were performed as described in section 2. (a) L2A effectively blocks binding of [^{125}I]NGF to the recombinant TrkA extracellular domain. [^{125}I]NGF, [^{125}I]BDNF, [^{125}I]NT-3, and [^{125}I]NT-4 were preincubated with concentrations of L2A ranging from 0 M, 1.86×10^{-8} M to 3.72×10^{-5} M and subsequently added to the immobilized recombinant extracellular domains of TrkA ([^{125}I]NGF) or TrkB ([^{125}I]BDNF, [^{125}I]NT-3, and [^{125}I]NT-4). After equilibrium binding had been reached, the amount of [^{125}I]neurotrophin bound to the respective domain was determined. Analogous experiments were performed with the N-terminally truncated L2A(−3) peptide, but only the curve for [^{125}I]NGF is shown here since for the other [^{125}I]neurotrophins the curve were indistinguishable from the ones obtained with L2A. All data points are means of duplicates. (b) L2B effectively blocks binding of [^{125}I]BDNF, [^{125}I]NT-3, and [^{125}I]NT-4 to the recombinant TrkB extracellular domain. The experiments were performed in an identical fashion as in (a) except that L2B was used as an inhibitor. All data points are means of duplicates.

The column was subjected to two elution steps designed to yield information on the precision of the observed interaction. In the first elution step, a heterologous peptide corresponding to the second LRM of rat TrkB (L2B) was applied in high excess to assess the specificity of the interaction between [^{125}I]NGF and L2A. L2B shares 13 identical amino in a total of 24 with L2A making it the ideal tool for such a purpose. No detectable amounts of [^{125}I]NGF eluted from the column after the addition of L2B (Fig. 1). In the second elution step an excess of soluble L2A was applied to the column. If the binding of [^{125}I]NGF to the column had been actually due to specific interaction with the immobilized L2A peptide, this step should lead to a specific, competitive elution of the radioligand. In fact, the virtually all of the [^{125}I]NGF that had bound to the column eluted in a distinct peak when the soluble L2A peptide was added (Fig. 1) proving that the L2A peptide on the column indeed accounts for the observed binding phenomena. The second LRM of TrkA therefore embodies a binding site of high specificity enabling it to solely bind NGF among the array of highly homologous neurotrophins.

Further proof of the specificity of the interaction between NGF and L2A was obtained by loading [^{125}I]BDNF onto an

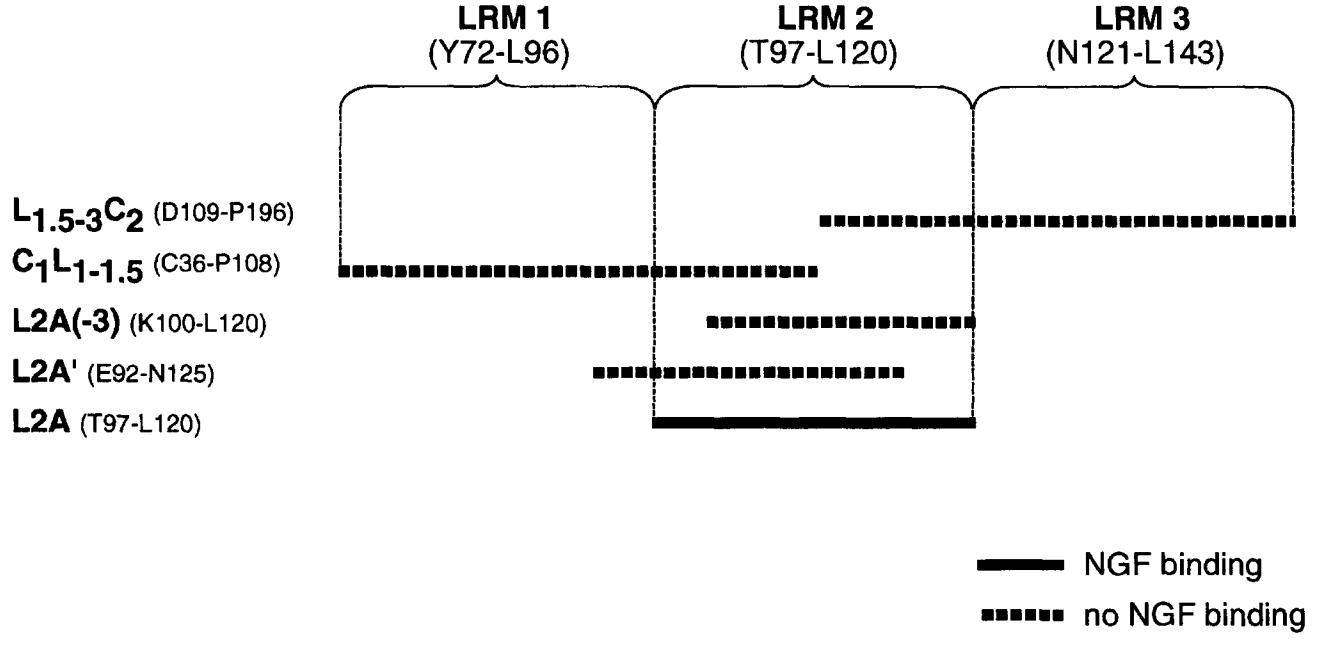

Fig. 3. Importance of phasing for the activity of L2A. The entire 24 amino acids of the second LRM of TrkA (L2A) are required for proper binding of NGF. Proteins disrupting this LRM from either side as well as peptide truncated by three amino acids at the N-terminus [L2A(−3)] were inactive. A protein expressed in the boundaries suggested by Kobe and Deisenhofer [17] (L2A') was also unable to bind NGF.

Fig. 4. Alignment of the second leucine-rich motifs of TrkA and TrkB receptors from different species. The rat TrkA [22] and rat TrkB [23] sequences correspond to the ones of the L2A and L2B peptides used in this work. The sequences of human TrkA, mouse TrkB, and chick TrkB were taken from Martin-Zanca et al. [19], Klein et al. [12], and Dechant et al. [3], respectively. The localizations of the motifs within the respective proteins are indicated by the numbers on either side of the sequences. Residues which are identical between all species in a given Trk-type are shown in bold upper-case, residues which vary are shown in standard lower-case. In the consensus sequences a '*b*' indicates a basic residue, an '*h*' a hydrophobic residue, an '*s*' a residue with a small side chain, and an 'x' any residue. The last line represents the general consensus sequence of the second LRM of TrkA and TrkB with the information from all species combined. The hydrophobic residues characteristic of a leucine-rich motif are underlined.

L2A column as described above for [^{125}I]NGF. In contrast to [^{125}I]NGF, however, the entire amount of [^{125}I]BDNF radioactivity was found in the flow through (Fig. 1). Similar experiments were conducted with [^{125}I]NT-3 and [^{125}I]NT-4 leading to the same results as with [^{125}I]BDNF. Therefore, the second LRM of TrkA indeed represents a binding site reserved exclusively for NGF.

After this novel approach had turned out to work for L2A, it was utilized to address the question of neurotrophin binding within the LRM_3-cassette of TrkB. TrkB, in contrast to TrkA, has three different ligands, BDNF, NT-3 and NT-4 [8,24] and therefore represents a much more complex system. Surprisingly, [^{125}I]BDNF actually bound to the L2B column with an affinity comparable to that of the interaction between L2A and NGF. Analogous experiments using L2B columns were conducted with [^{125}I]NT-3 and [^{125}I]NT-4 leading to similar results as with [^{125}I]BDNF even though binding of these neurotrophins to the covalently immobilized neurotrophin was much less pronounced than that of [^{125}I]BDNF. We could therefore identify the second LRM as a potent binding site for all three TrkB ligands. This is highly interesting since a mere 24 (or eleven considering that 13 are identical to that of the second LRM of TrkA) are capable of specifically recognizing three different neurotrophins.

Even though these results are highly satisfying in qualitative terms, i.e. in terms of the underlying highly specific biochemical mechanism, the observed binding phenomena apparently can not quantitatively account for the binding affinity observed in vivo [3,22,25,34] or using recombinant receptor proteins [39]. It must therefore be assumed that the capacity of the columns was aggravated by the fact that a large percentage of the peptides was either coupled to the matrix in a way that abolished binding affinity or was simply folded in an incorrect fashion [4,10,20]. Some peptides only exhibit a weak tendency to adopt a defined tertiary structure so that in a steady state balance only a small number of peptides exists in an active form at a given time point [10]. Furthermore, there are prominent examples of peptides that need the support of carrier proteins in order to acquire the correct conformation and thus function properly [33,37]. In the case of the L2A and L2B peptides this might well be the case since we have previously shown binding of [^{125}I]NGF with high affinity ($K_d \approx 1$ nM) to a recombinant fusion protein of the *E. coli* maltose binding protein (MBP) and the second LRM of TrkA. Most likely a combination of the phenomena described above accounts for the low capacity of the peptide columns observed in our experiments.

To obtain more precise information on the role these binding modules play in the biochemistry of TrkA and the TrkB receptors, the effect of these peptides (L2A, L2A(−3), and L2B) in inhibiting the binding of the different neurotrophins to the immobilized recombinant TrkA and TrkB extracellular domains was investigated as described in section 2. In these experiments the L2A peptide led to a pronounced inhibition of [^{125}I]NGF binding to TrkA but it had no effect on the binding of [^{125}I]BDNF, [^{125}I]NT-3 and [^{125}I]NT-4 to TrkB even when present at very high concentrations (Fig. 2a). The N-terminally truncated L2A(−3) peptide, however, had practically no effect on [^{125}I]NGF binding to TrkA even when present at a 1000fold excess over the TrkA extracellular domain (Fig. 3).

We also tested the ability of [^{125}I]NGF to bind to recombinant fusion-proteins disrupting the second LRM of TrkA from either side [38] as well as to a fusion-protein with the LRM boundaries suggested by Kobe and Deisenhofer [17], which means that the entire 24 residues of L2A are shifted five amino acids toward the N-terminus of TrkA. None of these proteins exhibited significant binding affinity for [^{125}I]NGF (Fig. 3). This supports our hypothesis that not a certain set of individual residues within the second LRM of TrkA, but much rather the entire structure is required for efficient ligand binding and that correct phasing is a prerequisite for a leucine-rich motif to function properly.

In conformity with its binding capacities, the L2B peptide showed a wider range of inhibitory effects with respect to the different neurotrophins. It inhibited the binding of all three TrkB ligands, BDNF, NT-3 and NT-4, to the recombinant extracellular domain of TrkB. It had no effect on the binding of NGF to TrkA (Fig. 2b).

These results clearly indicate that the second LRM plays a significant role in the binding of neurotrophin to TrkA as well as TrkB. However, the efficacy of the interference of the L2A or L2B peptides with the binding of the various neurotrophins was not as great as could be expected from previous experiments involving recombinant proteins [39]. Calculation of the inhibition constants for the interaction between the peptides and the neurotrophins yielded K_i values in the range of 2×10^{-8} M to 1×10^{-7} M which is at least a factor 20 short of the affinities observed on recombinant receptors as well as on cells ectopically expressing TrkA [9,22] or TrkB [3,31] receptors. This supports our interpretation of the low capacity observed with L2A and L2B affinity columns, i.e. that only a small portion of the peptides is folded in the same fashion as the corresponding region within the entire receptor protein.

The biochemical complexity displayed by these short binding site peptides corroborates the notion that homologous amino acid sequence modules can, simply by exchanging a few crucial residues during evolution, assume decidedly different and highly specific functions. It is also consistent with this evolu-

tionary view that, in the Trk-type receptor most highly conserved between species, TrkC, the second LRM exhibits the highest degree of conservation of all three LRMs in the LRM_3-cassette with variations at only two of 24 positions. Fig. 4 shows an alignment of the sequences of the second leucine-rich motifs of TrkA and TrkB receptors from different species. There is a single conservative exchange between rat and human TrkA. It will be highly interesting to see if this conservation is also preserved when TrkA sequences from species evolutionarily more distant to mammalians become known since this would give important clues to how binding specificity is achieved in this receptor. The second LRM of TrkB shows a higher degree of sequence variation. As a result of the complexity inherent in having to deal with three different ligands, it is impossible at this point to identify specific 'binding residues' within the second LRM of TrkA or TrkB. It seems more likely that the entire LRM structure in combination with distinct sets of residues account for the high biochemical complexity of these short binding motifs.

The biological importance of the second LRM of Trk receptors as a major neurotrophin binding site is underscored by in vivo experiments recently performed by our group where L2A peptides led to the specific degeneration of NGF dependent neurons in the rat brain (C. Humpel, J.M. Windisch, and R. Schneider, unpublished observations).

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