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Circular dichroism and crosslinking studies of the interaction between four neurotrophins and the extracellular domain of the low-affinity neurotrophin receptor

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Abstract

Interactions between the purified recombinant receptor extracellular domain (RED) of the human low-affinity neurotrophin receptor (LANR) and recombinant human brain-derived neurotrophic factor, neurotrophin-3 (NT-3) and neurotrophin-4/5 have been studied by chemical crosslinking and circular dichroism. Conformational changes subsequent to binding have been shown by these procedures. First, relative affinities of the neurotrophins for RED were determined by binding competition assays in which radioiodinated nerve growth factor (NGF) from mouse submaxillary gland was crosslinked to RED in the presence of varying amounts of unlabeled neurotrophin competitors. RED bound each of the 3 recombinant human neurotrophins with affinities that were indistinguishable from authentic mouse NGF. These results are the first measurement of binding of the neurotrophin family to their common receptor using purified components. In order to study the effect of binding on the conformation of the proteins, CD measurements were made before and after mixing neurotrophins and RED, as had previously been done with NGF and RED (Timm DE, Vissavajjhala P, Ross AH, Neet KE, 1992, *Protein Sci 1*:1023–1031). Similar changes in CD spectra occurred upon combination of each of the neurotrophins and RED, with negative changes near 220–225 nm and positive changes near 190–200 nm; however, significant differences existed among the various neurotrophin–RED difference spectra. The NT-3/RED complex showed the largest spectral change and NGF the smallest. Thus, specific conformational changes in secondary structure of neurotrophin, RED, or both accompany the binding of each neurotrophin to the extracellular domain of the LANR.

Keywords: circular dichroism; conformational change; low-affinity NGF receptor; nerve growth factor; neurotrophin; receptor

The neurotrophins are a family of target-derived trophic factors required for the development and survival of specific neuronal populations (Levi-Montalcini, 1987). This family of homologous proteins includes nerve growth factor, brain-derived neurotrophic factor, neurotrophin-3, and neurotrophin-4/5 (Ebendal, 1992). The overall sequence identity within this family is about 50% with any pair being about 55–65% similar. The structure of NGF, the first neurotrophin to be discovered (Levi-Montalcini & Booker, 1960), has been solved by X-ray crystallography; the monomeric unit is comprised of 3 pairs of β-strands connected by 3 β-hairpin regions and 1 region of 3 consecutive reverse turns (McDonald et al., 1991).

The action of neurotrophins on nerve cells is mediated through signal transduction pathways initiated by the binding of these factors to high-affinity receptors (Sutter et al., 1979), having dissociation constants in the range of 10^{-11} M. TrkA (or $p140^{TRK}$), a receptor protein tyrosine kinase, is a component of the high-affinity NGF receptor (Kaplan et al., 1991; Klein et al., 1991). TrkB (Squinto et al., 1991; Soppet et al., 1991) and TrkC

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Abbreviations: BDNF, brain-derived neurotrophic factor; BSA, bovine serum albumin; EDAC, ethyldimethylisopropylaminocarbodiimide; EGF, epidermal growth factor; LANR, low-affinity neurotrophin receptor; NT-3, neurotrophin-3; NT-4/5, neurotrophin-4/5; NGF, nerve growth factor; NTF, neurotrophic factor; RED, receptor extracellular domain; TNF-R, tumor necrosis factor receptor.

(Lamballe et al., 1991), homologous Trk family members, are major receptors for BDNF and NT-3, respectively. Interestingly, NT-4/5 may interact with both TrkA and TrkB (Berkemeier et al., 1991; Hallbook et al., 1991; Ip et al., 1992). The low-affinity NGF receptor (LANR or p75) is a glycoprotein composed of 211 extracellular, 33 transmembrane, and only 155 cytoplasmic residues (Johnson et al., 1986) with an apparent molecular mass of 75 kDa. LANR is co-expressed with TrkA by many NGF-dependent neurons (Ebendal, 1992). LANR on the surface of PC12 cells has been shown to bind to each of the neurotrophins with similar affinity (about 10^{-9} M) but with different kinetics (Rodriguez-Tebar et al., 1990, 1992). However, a consensus on the role of the LANR in high-affinity NGF binding has not yet been reached (Chao, 1992; Meakin & Shooter, 1992).

Although the LANR has no associated enzymatic activity, its involvement in the neurotrophic response is suggested by the following observations (reviewed by Ross, 1991; Chao, 1992; Meakin & Shooter, 1992). High-affinity neurotrophin binding and responsiveness has resulted from transfection of LANR into particular cell lines (Hempstead et al., 1989; Matsushima & Bogenmann, 1990; Pleasure et al., 1990), LANR has been detected in high-affinity complexes crosslinked to NGF (Green & Greene, 1986), and targeted disruption of the LANR gene in mice by homologous recombination was found to impair sensory nervous function (Lee et al., 1992).

The recombinant receptor extracellular domain of the LANR has been characterized following expression in insect cells (Vissavajjhala & Ross, 1990). The RED is an asymmetric, nonglycosylated molecule with little or no α-helix or β-sheet (Vissavajjhala & Ross, 1990; Timm et al., 1992). Mixing of NGF and RED resulted in a small alteration of the far-UV CD spectra, indicating a change in secondary structure consequent to interaction (Timm et al., 1992). Despite the overall similarity in neurotrophin sequence of about 50%, significant differences in thermodynamic stabilities and CD spectra of the neurotrophins have been reported (Radziejewski et al., 1992; Timm & Neet, 1992). Hence, it was of interest to compare the binding and conformational changes of the family of neurotrophins with RED. We report here that RED has similar affinity for NGF, BDNF, NT-3, and NT-4/5, and that similar, but nonidentical, CD spectroscopic changes result from the combination of RED with each neurotrophin.

Results

In order to measure changes in conformation by CD, demonstration of binding of each neurotrophin to the RED was necessary. Competition for crosslinking with NGF was chosen because other methods did not work well for RED (A.H. Ross, unpubl. obs.). The initial experiments, thus, were designed to demonstrate the binding of the neurotrophins to a purified receptor component, i.e., to the extracellular domain of the LANR.

Competition for crosslinking

As shown previously (Vissavajjhala & Ross, 1990), ^{125}I-NGF forms a stable complex with RED that is readily detected following crosslinking with EDAC as a doublet migrating on SDS polyacrylamide gels. The apparent molecular weight of the complex seen here, 43 kDa (Fig. 1A), is slightly lower than that reported previously (Vissavajjhala & Ross, 1990), probably because of different electrophoresis buffer systems used. Recombinant human BDNF, NT-3, and NT-4/5 compete with ^{125}I-NGF crosslinking to RED in a manner similar to unlabeled, control mouse NGF (Fig. 1B–D).

The crosslinked complex is specific to RED and the neurotrophins, because the reactions were carried out in the presence of 0.1% BSA and the complex was not apparent when RED or EDAC was omitted (Fig. 1A–D, lanes 13, 14). The amount of labeled NGF in the complex was quantitated by phosphorescence imaging and plotted as a function of unlabeled neurotrophin concentration (Fig. 2). The radiolabeled NGF is displaced equally well by each of the unlabeled neurotrophins, suggesting similar affinities. Little displacement of ^{125}I-NGF is observed below a concentration of 80 nM for the competitor neurotrophins; however, a sharp displacement occurs above this concentration. The concentration for 50% displacement (IC_{50}) estimated from semi-log plots (Fig. 2) of the quantitated radioactivity associated with the complex is about 200 nM for each neurotrophin. Due to the concentration of RED (relative to the K_D) needed to obtain a quantitatable autoradiographic band, this IC_{50} value would be an overestimate of the true K_D. This possibility was tested by attempting the crosslinking experiment at a lower total RED concentration. A 5-fold reduction of the RED concentration resulted in a 5-fold decrease in the IC_{50} of the NGF displacement curve (data not shown). This dependence of IC_{50} on receptor concentration indicates that neurotrophin binding to RED is near stoichiometric under these assay conditions. However, data quality and quantitation of the weak autoradiographic bands were reduced significantly at the lowered RED concentration and are not shown here. The true K_D for the RED/neurotrophin complex after correction for these factors is likely to be in the range of that measured for neurotrophin binding to the LANR on the cell surface (see Discussion).

A radiolabeled band with an apparent molecular weight consistent with the neurotrophin dimer (~26 kDa) also appears at the higher concentrations of neurotrophin used in the competition assays. The concentration-dependent appearance of this band is an indication that heterodimer formation between NGF and the other neurotrophins may occur. Further experiments are required to verify this possibility.

CD of the neurotrophins and RED

The individual CD spectra for the NGF, BDNF, NT-3, and RED have been reported (Radziejewski et al., 1992; Timm et al., 1992; Narhi et al., 1993). The NT-4/5 spectra are similar but distinct from the other neurotrophins (Fandl et al., 1994; N. Panayotatos, pers. comm.). NGF has a single minimum and maximum at 208 nm ($-6{,}000$ deg·cm^2/dmol) and 190 nm (6,000 deg·cm^2/dmol), respectively. The BDNF, NT-3, and NT-4/5 CD spectra contain these features but differ from the NGF CD spectra by an additional positive signal between 225 and 235 nm and an additional negative signal at 195 nm (Radziejewski et al., 1992). Despite significant, small differences in the shapes of these spectra, estimates indicate that the neurotrophins have similar secondary structure contents, composed primarily of β-sheet (Radziejewski et al., 1992). The CD spectrum of RED, on the other hand, has a maximum at 225 nm of 2,000 deg·cm^2/dmol and a minimum at 197 nm of $-27{,}000$ deg·cm^2/dmol (Timm et al., 1992). This RED spectrum is most similar to the random

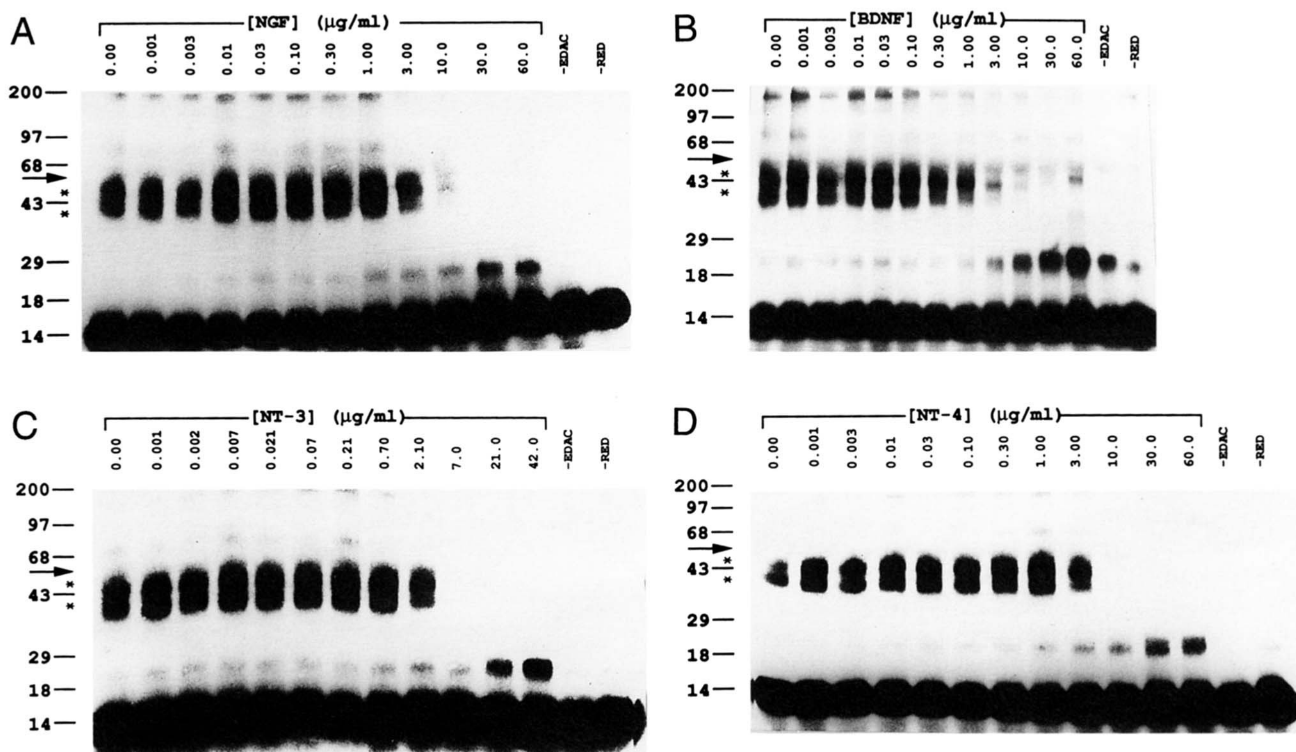

Fig. 1. Crosslinking competition assays. Autoradiograms of crosslinking competition assays using the indicated concentrations of unlabeled (**A**) NGF, (**B**) BDNF, (**C**) NT-3, and (**D**) NT-4/5 were constructed as described in Materials and methods. The positions of the RED/^{125}I-NGF complex (*), carrier BSA (arrow), and prestained standards (BRL) are indicated.

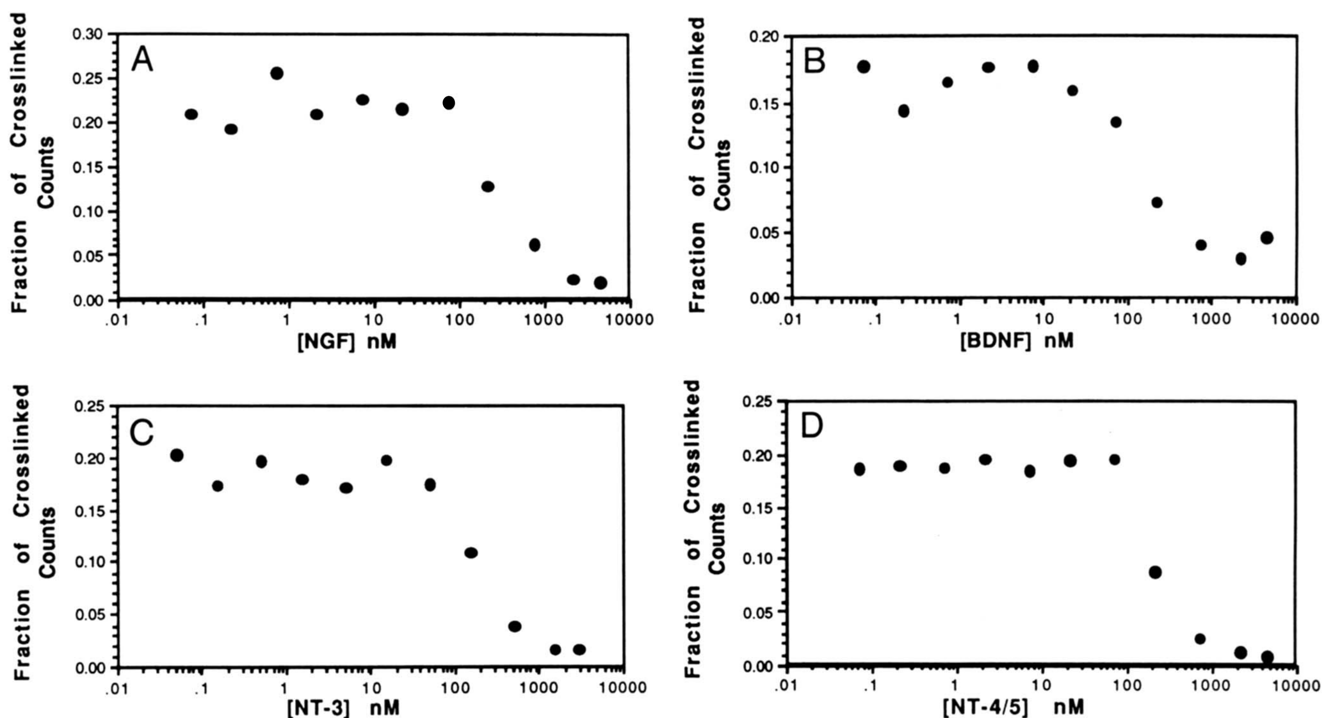

Fig. 2. Quantitation of the competition assays. The experiments of Figure 1 were quantitated using a phosphorescence imager (see Materials and methods) and semi-log plots were constructed. Background radioactivity at the position of the RED/^{125}I-NGF complex in control lanes (13 and 14) lacking RED and EDAC was subtracted, and data were normalized by dividing the cpm associated with the RED/^{125}I-NGF complex by the sum of cpm associated with free ^{125}I-NGF and the complex.

coil signal of protonated polylysine (Brahms & Brahms, 1980) or the deconvoluted β-turn spectra (Compton & Johnson, 1986).

CD of the complex

Changes in CD spectra in the far UV were observed upon mixing of RED with each individual neurotrophin. The spectrum at these wavelengths is particularly sensitive to the secondary structure of proteins. Mixing of NGF and RED had previously been shown to result in slightly more negative ellipticity between 220 and 235 nm and decreased negative ellipticity below 220 nm (Timm et al., 1992). As expected from the distinct spectra of the individual neurotrophins, the summation of the RED spectrum with BDNF, NT-3, and NT-4/5 spectrum (unmixed, Fig. 3, solid lines) differs from that for NGF, particularly the positive peak at about 228 nm previously reported for BDNF, NT-3, and NT-4/5, individually (Radziejewski et al., 1992; N. Panayotatos, pers. comm.). Differences were observed when the CD spectra for RED plus BDNF, NT-3, or NT-4/5 were recorded with the receptor and neurotrophins separated in a tandem cuvette compared to the spectra following mixing of the components (Fig. 3, solid lines versus dotted lines). In each case, there was a decreased ellipticity of the positive peak at about 225–230 nm and a less negative ellipticity of the trough at 195–205 nm after mixing. Therefore, a conformational change accompanies binding of each of these neurotrophins to RED that appears to have overall similar aspects. The difference spectra, ΔCD (separate subtracted from mixed components), for all 4 neurotrophins are generally similar (Fig. 4) and highlight the fact that a conformational change occurs subsequent to complex formation. On closer examination, however, differences appear among the neurotrophins in the magnitude and position of the ΔCD (Fig. 4; Table 1). We conclude that binding of a neurotrophin to RED results in a significant secondary structure change in RED, the neurotrophin, or both components, which produces a change in the CD spectra of 10–20% at 205 nm and up to 80% change in the smaller signal at 225 nm (Table 1). These changes appear to be significantly different among the 4 neurotrophins.

Several complications to generation of the CD difference spectra were considered. Mixing of RED with several proteins not expected to bind the receptor (ribonuclease, cytochrome *c*, or insulin) and mixing of proteins that do not interact (αNGF with γNGF) did not result in altered CD spectra (Timm et al., 1992). Furthermore, the results of the mixing experiments could not be simulated by simple summations where the RED and neurotrophin spectra were reduced by constant fractions (data not shown). The low buffer and salt concentrations used to extend the transparency of the solutions to the far UV in CD measurements did not affect binding competition assays using unlabeled NGF (data not shown). Greater than 90% receptor occupancy by the neurotrophins was calculated from limit value of K_D (10 nM), obtained from the results of the crosslinking competition assays (see below), and the final concentrations of 75 nM RED and 112 nM neurotrophin used in CD measurements. Furthermore, the molar ΔCD spectrum for NGF/RED was essentially the same here as in our earlier experiments at 6-fold higher concentrations (Timm et al., 1992). We conclude that the observed changes in CD are due to specific interactions between RED and neurotrophin and are not attributable to an artifactual decrease, weaker binding, or nonspecific changes of the components.

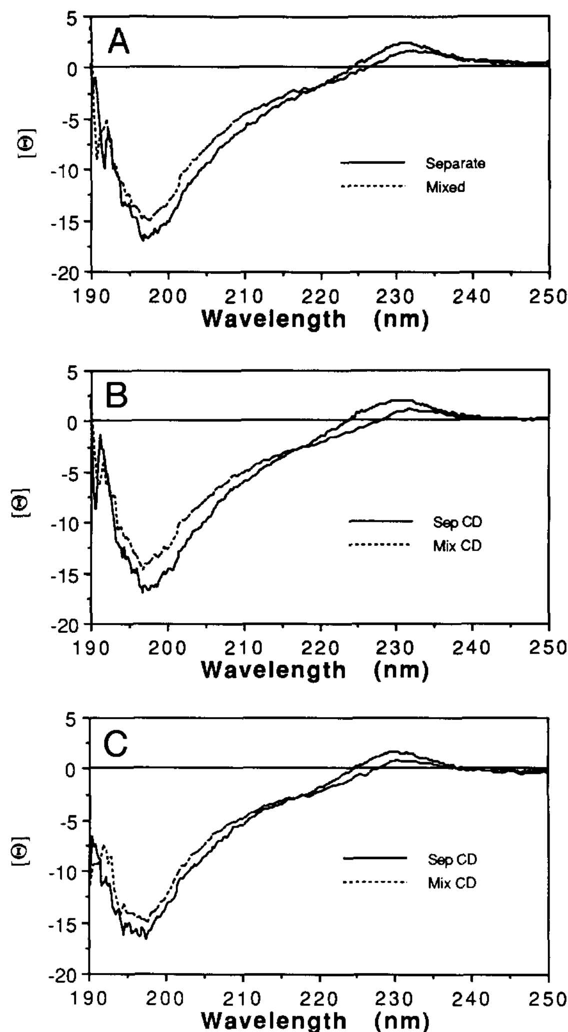

Fig. 3. CD spectra of the complex compared to the individual components. The mean residue ellipticity (deg $cm^2/dmol \times 10^{-3}$) of the neurotrophins and RED are shown as the sum of the separate components (solid line) and following mixing of the RED and neurotrophin (dashed line). **A:** BDNF. **B:** NT-3. **C:** NT-4/5. See Materials and methods for details. NGF was reported in Timm et al. (1992). Final RED concentration was 75 nM and neurotrophins were 112 nM (calculated as dimer).

Discussion

Although the exact role of the LANR in forming the high-affinity receptor is not clear (Kaplan et al., 1991; Klein et al., 1991), several functions have been suggested for the physiological function of LANR (see review by Chao, 1992). These possibilities include concentration and presentation of neurotrophins (Taniuchi et al., 1988), G-protein-coupled signaling (Feinstein & Larhammer, 1990), retrograde neurotrophin transport (Johnson et al., 1987), discrimination between neurotrophins (Rodriguez-Tebar et al., 1992), association with signaling components or Trk substrates (Ohmichi et al., 1991), association with other kinases (Volonte et al., 1993), cellular specificity (Ip et al., 1993), programmed cell death (Rabizadeh et al., 1993), and neuronal excitation (Palmer et al., 1993). Such receptor-mediated responses may involve conformational changes induced by ligand binding (Yarden & Ullrich, 1988).

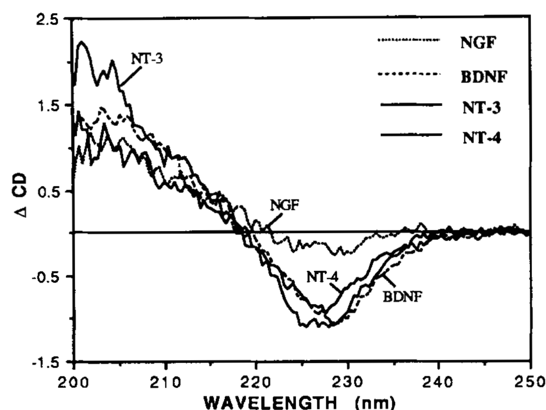

Fig. 4. CD difference spectra (ΔCD) of the complex. CD difference spectra (mean residue ellipticity, deg cm^2/dmol × 10^{-3}) were obtained for NGF, BDNF, NT-3, and NT-4/5 by subtraction of the digitized spectra from the experiments in Figure 3 (sum of mixed spectra minus spectra of separated components). The average of 3 difference spectra for each neurotrophin is presented. See Materials and methods for details.

Receptor crosslinking assays indicate recombinant human BDNF, NT-3, and NT-4/5 bound RED with similar affinities as mouse NGF. This result with the purified recombinant extracellular domain confirms studies demonstrating that LANR on the cell surface binds all 4 neurotrophins with similar affinities (Rodriguez-Tebar et al., 1990, 1992; Hallbook et al., 1991; Squinto et al., 1991). Mutagenesis (Yan & Chao, 1991; Baldwin et al., 1992) and proteolysis studies (Vissavajjhala et al., 1992) have demonstrated that the integrity and interaction of the 4 cysteine-rich repeat domains are necessary for NGF binding to the LANR. The IC_{50} values presented here and in previous crosslinking studies (Vissavajjhala & Ross, 1990) were obtained with high RED concentration and, therefore, represent upper limits to the true dissociation constants for RED and the neurotrophins. Correction of the IC_{50} by a term (1 + [^{125}I-NGF]/K_{NGF}) for competitive binding and by subtraction of [RED]/2 to account for the stoichiometric binding conditions (Williams & Morrison, 1979) yields a value of about 10 nM for the corrected

Table 1. *Characteristics of the RED/neurotrophin CD difference spectra*

	205 nm		225 nm	
Neurotrophin	ΔCD ± SD[a]	% Change[b]	ΔCD ± SD[a]	% Change
NGF	1.0 ± 0.2	10	−0.2 ± 0.1[c]	14
BDNF	1.3 ± 0.2	13	−0.8 ± 0.1	57
NT-3	1.9 ± 0.2[d]	20	−1.1 ± 0.2	78
NT-4/5	1.0 ± 0.1	11	−0.8 ± 0.1	65

[a] ΔCD (deg cm^2/dmol × 10^{-3}) calculated from Figures 3 and 4. $P < 0.01$ for all ΔCD values being significantly different from zero.
[b] Percent change calculated as ΔCD/CD of separate summed spectra × 100.
[c] $P < 0.001$ compared to the other 3 neurotrophin values at the same λ.
[d] $P < 0.05$ compared to the other 3 neurotrophin values at the same λ.

K_D. This value still represents a maximal estimate of the dissociation constant but is in reasonable agreement with that for LANR (K_D about 1 nM) measured on the cell surface (Sutter et al., 1979; Vale & Shooter, 1985; Woodruff & Neet, 1986; Rodriguez-Tebar et al., 1990, 1992; Squinto et al., 1991; Ip et al., 1992), measured with intact solubilized receptor (EC_{50} about 30 nM) (Marano et al., 1987), or measured with naturally occurring, truncated LANR (EC_{50} = 15 nM) (Zupan et al., 1989). This agreement between dissociation constants for binding to the purified, soluble extracellular domain and the intact receptor suggests the apparent affinity is not significantly affected by components in the cell membrane or by the receptor's cytosolic domain.

The changes in the far UV CD that occur upon combining RED and the neurotrophins indicate that conformational changes accompany binding. Binding of all 4 neurotrophins causes changes in the same region of the CD spectrum, thus suggesting that common structural features are shared between complexes of RED and the various neurotrophins. The shape and intensities of the observed differences in CD vary, depending on which neurotrophin is mixed with RED (Figs. 3, 4); values at 2 select wavelengths are given in Table 1 for comparison. These qualitatively distinct spectra indicate that the conformational change is different for complex formation with each neurotrophin (see below). The NGF/RED complex appears most different, i.e., NGF produces the smallest ΔCD between 220 and 230 nm (Fig. 3; Table 1). At 205 nm, NT-3 clearly produces the largest difference peak compared to the 3 other neurotrophins. The spectra of the isolated neurotrophins tend to correspond with the wavelength of the ΔCD of the complex (Fig. 3). NGF has the most negative ellipticity at 225–230 nm with no positive peak (Timm & Neet, 1992), and the ΔCD for RED/NGF is the smallest at this wavelength. The NT-3 CD spectra have the most negative trough at 200–205 nm (Radziejewski et al., 1992) and the RED/NT3 complex has the largest ΔCD at these wavelengths, compared to the other neurotrophins. Similar, but weaker, correspondence occurs in the 220–230-nm region for BDNF, NT-3, and NT-4/5. On the other hand, these wavelengths of ΔCD peaks are also the same regions (220–230 and 195–205 nm) in which the CD spectra of RED alone have a pronounced maximum and minimum (Timm et al., 1992). In binding studies, BDNF showed the slowest rates of association and dissociation of the 3 neurotrophins (Rodriguez-Tebar et al., 1990, 1992), indicating that the conformational change observed here may be related but is not directly correlated with the rate of binding.

We attempted to quantitate the change in secondary structure by the method of convex constraint algorithm (Perczel et al., 1991, 1992a), which has been well utilized for proteins of high β and random structure with CD spectra from 195 to 240 nm (Perczel et al., 1992b). The results with the individual proteins were consistent with previous estimates that random secondary structure dominated the RED spectra (Timm et al., 1992) and that the neurotrophins had mainly β structures with little α-helix (Radziejewski et al., 1992; Timm & Neet, 1992; Narhi et al., 1993); the convex constraint algorithm method also predicted large percentages of aromatic/disulfide contributions to the CD spectra. Overall the analysis of neurotrophin/RED complex formation suggested a shift from unordered to ordered structure, consistent with the qualitative observation, with variations of a few percent among the 4 neurotrophins. Calculations showed apparent decreases of 3–4% in random structure and 2–6% in

β-turns/parallel β structure upon mixing with an increase of 3–8% in antiparallel β-strands; aromatic/disulfide contributions either increased or decreased by less than 5%. However, the analyses were not entirely satisfactory because the separate (additive) percentages of components were not well predicted by contributions of pure spectra from individual proteins and because the content of antiparallel β structure was significantly low in all separate and mixed spectra relative to individual spectra or to the crystal structure. Therefore, we do not have sufficient confidence in these analyses to report the exact values from the convex constraint algorithm. Nevertheless, we conclude that the magnitude of the changes seen in the difference spectra (Fig. 4) are consistent with increases in ordered secondary structure between 4 and 10%. One cannot determine whether the CD changes upon complex formation are most likely to be in the neurotrophin or in the RED.

Conformational changes with significant biological effects frequently consist of large relative domain movements or reorientation of loops (Kempner, 1993); many of these do not involve significant change in net secondary structure. The magnitude of ΔCD with the neurotrophins and the LANR RED is similar to that observed upon mixing of EGF (Greenfield et al., 1989) or insulin (Schaefer et al., 1992) with each respective receptor extracellular domain. In the case of EGF, the extracellular domain complex is then capable of oligomerizing (Lax et al., 1991). The interaction of ligand with these latter receptors, of course, triggers protein tyrosine kinase activity of their cytosolic domains.

The altered CD spectra subsequent to complex formation may be due to a conformational change occurring in the neurotrophin, the RED, or both molecules. A model depicting these possibilities is shown in Figure 5. Because LANR is a member of the NGF receptor/TNF-R superfamily (Smith et al., 1990), the model is based upon the crystal structure of the TNFβ-sTNF-R55 complex (Banner et al., 1993). sTNF-R55 has a high content of loops, extended β, β bends, and β meander structures, consistent with the CD of the LANR RED that predicts mainly β structure and random coil. Both TNF-R and LANR have 4 repeating cysteine-rich domains of about 40–50 residues each. sTNF-R55 has been shown to bind to TNFβ by binding in the shallow surface groove between subunits in the trimeric TNFβ (Banner et al., 1993). A similar arrangement is suggested with NTF and LANR in Figure 5.

If the conformational change occurs in the neurotrophin (Fig. 5, path B), a likely region is in the hairpin turn at residues 25–36, which has been implicated in LANR binding (Ibanez et al., 1992) and contains several substitutions and insertions in the neurotrophin family (Ebendal, 1992). Small changes in the conformation of TNF (which has no structural similarity to NGF) upon binding to sTNF-R55 have been demonstrated from X-ray analysis of the complex; 3 short loops (105–110, 46–51, and 83–94) attain more order or undergo changes in ϕ, ψ angles (Banner et al., 1993). Because the neurotrophins have different initial states defined by the CD spectra of the isolated neurotrophins (Radziejewski et al., 1992; Fandl et al., 1994; D.E. Timm, unpubl. obs.; Fig. 3, solid lines; and discussion above), the various difference spectra (Fig. 4) might then reflect achievement of a similar final conformational state. However, the final spectra (dotted lines in Fig. 3) are distinct among the 4 neurotrophins, particularly between 190 and 200 nm, suggesting that the complexes do not all attain the same final conformation. This conclusion is supported by the estimates of secondary structure in the complexes, which were different for the 4 neurotrophins. Because neuronal responses show a restricted specificity in the presence of the LANR (Ip et al., 1993), the conformational state of the neurotrophin in the complex (Fig. 5, path B) could contribute to the selective presentation of the neurotrophin family by LANR to the Trk family for further signaling.

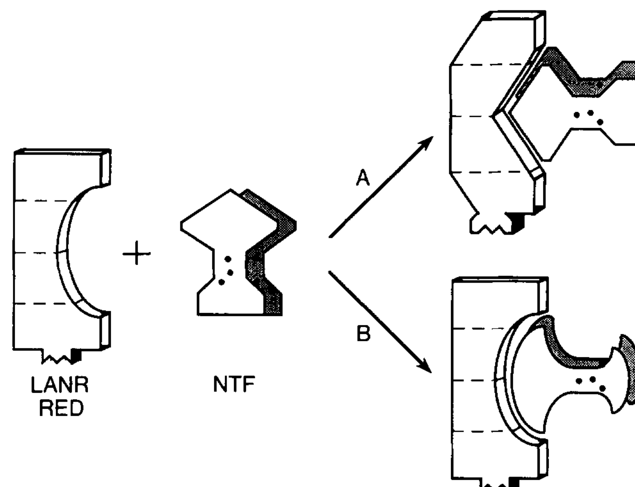

Fig. 5. A model for conformational changes in RED and neurotrophins. The receptor extracellular domain of the low-affinity neurotrophin receptor (LANR RED) can bind to each of the neurotrophins (NTF) with a conformational change occurring in the RED (path A) or in the NTF (path B) or in both (not shown). The RED is drawn as an elongated molecule interacting along the intersubunit dyad axis of the NTF, consistent with the homologous structure of the TNFβ–TNF-R55 complex crystal structure (Banner et al., 1993) in which the dashed lines suggest the cysteine-rich related domains of LANR RED; the molecule is truncated at the membrane (residue 183) as indicated by the jagged line. The NTF is shown as a dimer (white and shaded subunits), consistent with the presumed stoichiometry, that interacts through the loops containing the residues 32, 34, and 95 as proposed (Ibanez et al., 1992); the dots indicate the disulfide bonds for orientation purposes. See text for discussion.

On the other hand, flexibility in the RED molecule (Fig. 5, path A) could accommodate different initial conformations of each of the neurotrophins and distinct final states of the complex. Conformational changes could occur in the RED backbone near the interface between the 2 proteins and be propagated to other parts of the molecule (as suggested in Fig. 5, path A) to influence transmembrane signaling or to enhance formation of receptor homodimers or heterodimers. The neurotrophin–RED difference spectra would then reflect an induced fit mechanism giving rise to multiple, distinct RED states and accounting for the difference in binding kinetics (Rodriguez-Tebar et al., 1990, 1992). Future studies will need to focus on the significance and potential role of these conformational changes in receptor signaling.

Materials and methods

Materials

The β subunit of NGF was purified from mouse submaxillary glands by the 7S oligomeric NGF procedure (Smith et al., 1968; Stach et al., 1977; Woodruff & Neet, 1986) and ran as a single

band of 13 kDa on SDS polyacrylamide gel electrophoresis. NGF was radioiodinated to a specific activity of 53 cpm/pg using lactoperoxidase (Calbiochem) and $Na^{125}I$ (Amersham) with modifications of established procedures (Sutter et al., 1979). Native and radiolabeled NGF showed full bioactivity (EC_{50} = 20 pM) in a PC12 cell neurite outgrowth bioassay. Recombinant human BDNF and NT-3 were provided by Amgen, Inc. (Thousand Oaks, California) and recombinant human NT-4/5 was provided by Regeneron, Inc. (Tarrytown, New York). All recombinant neurotrophins were characterized to be greater than 90–95% homogeneous by their manufacturer and ran in this lab as single bands on SDS electrophoresis with no immunocross-reactivity with a monoclonal antibody to NGF. Mouse NGF was used in this study because of its availability, but the affinity of human and mouse NGF (90% sequence identity) for receptor is essentially identical; therefore, the differences in the species of the neurotrophins were considered to be of little consequence. Human RED was produced in a baculovirus expression system, purified as described (Vissavajjhala & Ross, 1990), and ran as a doublet on SDS electrophoresis. EDAC (Bio-Rad) was dissolved immediately before use in distilled, deionized H_2O. All other reagents were purchased from Sigma (St. Louis, Missouri) or United States Biochemical (Cleveland, Ohio) as ultrapure grade.

Receptor binding competition assay

Receptor crosslinking to ^{125}I-NGF using EDAC was performed with modifications of published procedures (Taniuchi et al., 1986; Vissavajjhala & Ross, 1990). All reactions and incubations were performed at room temperature (22 °C) in 50 mM NaH_2PO_4, 50 mM Na_2HPO_4, 150 mM NaCl, pH 6.8, containing 0.1% BSA. Briefly, ^{125}I-NGF and RED were combined and added to a series of unlabeled neurotrophin dilutions resulting in final ^{125}I-NGF and RED concentrations of 1 and 180 nM, respectively. The samples were then equilibrated for 24 h in order to ensure equilibration of the binding reaction (Rodriguez-Tebar et al., 1990, 1992). EDAC was then added to 30 mM; SDS sample buffer was added 12 min later; solutions were placed in boiling water for 5 min and then run on 10% polyacrylamide gels using a Tris-Tricine buffer system (Schagger & von Jagow, 1987). Gels were fixed in 40% methanol, 10% acetic acid, dried, and exposed to phosphorescence storage screens (Kodak) and/or XAR film (Kodak). Quantitations were performed using a Molecular Dynamics phosphorescence imager interfaced with a Gateway (IBM compatible) computer.

CD mixing experiments

Differences between the CD spectra in the far UV of mixed and separate solutions of RED and neurotrophins were measured as previously described (Timm et al., 1992). RED (3.5 μg/mL) and neurotrophin solutions (4–6 μg/mL) in 5 mM NaH_2PO_4, 5 mM Na_2HPO_4, pH 6.8, were placed in separate 4.37-mm-pathlength cells of a tandem cuvette (Helma). Spectra were then recorded as an average of 6 scans with subtraction of the buffer baseline using a Jasco J600 spectropolarimeter interfaced with a Wyse (IBM compatible) computer. The RED and neurotrophin solutions were then mixed by inversion, incubated at room temperature for 15 min, and CD spectra recorded as above. CD difference spectra were generated by subtraction of the digitized spectra recorded prior to and following mixing.

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