Characterization of the recombinant extracellular domain of the neurotrophin receptor TrkA and its interaction with nerve growth factor (NGF)

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Abstract

Nerve growth factor (NGF) is the prototype of a family of neurotrophins that support important neuronal programs such as differentiation and survival of a subset of sympathetic, sensory, and brain neurons. NGF binds to two classes of cell surface receptors: p75^{LANR} and p140^{TrkA}. NGF binding to p140^{TrkA} initiates the neuronal signaling pathway through activation of the tyrosine kinase activity, which subsequently results in a rapid signal transduction through a phosphorylation cascade. To examine this crucial signaling step in more detail, the TrkA extracellular domain polypeptide (TrkA-RED) was overexpressed in Sf21 insect cells and purified to homogeneity. The recombinant TrkA-RED is a 70 kDa acidic glycoprotein with a pI of 5.1, and mimics the intact TrkA receptor for NGF binding with a dissociation constant, K_d , of 2.9 nM. Thus, the recombinant TrkA-RED is functionally competent and can be used to elucidate the interaction of NGF and TrkA receptor. Circular dichroism difference spectra indicated that, upon association of NGF with TrkA-RED, a minor conformational change occurred to form a complex with decreased ordered secondary structure. Interaction between NGF and TrkA-RED was also demonstrated by size exclusion chromatography, light scattering, and chemical crosslinking with evidence for formation of a higher molecular weight complex consistent with a (TrkA-RED)₂–(NGF dimer) complex. Association and dissociation rates of $5.6 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ and $1.6 \times 10^{-3} \text{ s}^{-1}$, respectively, were determined by biosensor technology. Thus, initiation of signaling may stem from NGF-induced receptor dimerization concomitant with a small conformational change.

Keywords: mutant; nerve growth factor; neurotrophin; receptor binding; receptor extracellular domain; recombinant; structure

The growth factor (NGF) and its homologous neurotrophic factors are members of the growing family of neurotrophins that play a profound pharmacological role in development and survival of neuronal cells (Snider, 1994). Five members of the neurotrophin family have thus far been isolated, with NGF as the prototype for this family. The other members include brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT3), and neurotrophin-4/5

(NT4/5); fish neurotrophin-6 (NT6) is the most newly discovered neurotrophin (Ip & Yancopoulos, 1996). The biologically active form of NGF is a 26.5 kDa homodimer in which the monomeric polypeptide contains 118 amino acids. The high degree of sequence homology (more than 50% across different neurotrophins) and the determination of the X-ray crystallographic structure of NGF (McDonald et al., 1991; Holland et al., 1994) and BDNF–NT3 heterodimer (Robinson et al., 1995) indicate that all neurotrophins have a similar three-dimensional structure.

The neurotrophins interact with two classes of cell surface receptors on responsive cells, p75 low-affinity neurotrophin receptor (p75^{LANR}) and the Trk family of protein tyrosine kinase receptors. (Trk refers to all three Trk receptors; a specific receptor subtype will be termed TrkA, TrkB, or TrkC.) All neurotrophins are capable of binding with similar affinities to p75^{LANR}, a distantly related member of the TNFR receptor family (Gruss & Dower, 1995). However, different neurotrophins selectively interact with their cognate Trk receptors and display specific sets of biological activities on peripheral and central nervous system neurons (Davies, 1994; Ibanez, 1994).

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Abbreviations: BDNF, brain-derived neurotrophic factor; CM5, carboxymethylated dextran matrix; DSS, disuccinimidyl suberate; EDC, N-ethyl-N'-(dimethylaminopropyl)carbodiimide; HBS, HEPES buffered saline; LANR, low-affinity neurotrophin receptor; NGF, nerve growth factor β subunit; NT3, neurotrophin-3; NHS, N-hydroxysuccinimide; NT4/5, neurotrophin-4/5; PC12, pheochromocytoma 12 cell line; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; Sf21, *Spodoptera frugiperda* 21 (insect cell line); TrkA-RED, TrkA receptor extracellular domain

Binding of neurotrophins to the extracellular domain of the Trk receptors initiates neuronal differentiation through activation of the receptor tyrosine kinase activity, resulting in a rapid increase in the phosphorylation of selective cellular substrates (Kaplan & Stephens, 1994; Chao & Hempstead, 1995). NGF displays both high- and low-affinity binding in sensory and sympathetic neurons and pheochromocytoma (PC12) cells (Woodruff & Neet, 1986; Chao, 1992; Meakin & Shooter, 1992). Binding studies showed that both TrkA and p75^{LANR} bind NGF with a K_d of about 10^{-9} M when they are expressed alone in non-neuronal cells such as NIH 3T3 fibroblasts or *Spodoptera frugiperda* 21 (Sf21) insect cells (Chao & Hempstead, 1995; Woo et al., 1995; Woo & Neet, 1996). In other studies, overexpression of Trk alone generated mostly binding of NGF at low affinity sites with less than 5% of the NGF at high-affinity sites (Jing et al., 1992). Changes in the relative levels of p75^{LANR} or TrkA receptors can influence the overall binding affinity for NGF (Hempstead et al., 1992; Benedetti et al., 1993).

The sites of interaction with receptors have been characterized in neurotrophins by site-directed mutagenesis or deletion. The NGF regions conferring TrkA specificity relative to TrkB all lie along one face of the NGF dimer, with residues from both protomers contributing to the putative TrkA binding site (Ibanez, 1994; McDonald & Chao, 1995). The TrkA binding sites also include both N- and C-termini (Bothwell, 1995). In contrast, the neurotrophin sites that distinguish TrkA from TrkC binding have been reported to be located in the central β strands (Urfer et al., 1997) and in distinct regions in the "north side" loops and the N-terminus (Kullander et al., 1997). Binding to the p75 receptor has been localized to neurotrophin sequences that are primarily at the top of the dimer, partly overlapping with the TrkA binding sites (Ibanez et al., 1992; Ibanez, 1994). These results are generally in agreement with the results of immunochemical mapping of p75 and TrkA binding sites (Nanduri et al., 1994). These arrangements could allow a single NGF dimer to bind p75 and TrkA simultaneously, thereby stabilizing the formation of a p75–TrkA heterodimer and providing a high affinity site for NGF (Bothwell, 1995). Two hypothetical models of potential p75–TrkA interactions have been proposed to accommodate the divergent results about the roles of p75 (Chao & Hempstead, 1995).

Trk family receptors share a unique mosaic of distinct structural modules in the extracellular domain (Schneider & Schweiger, 1991). In vitro binding assays with recombinant modules suggest that the LRM repeats (Windisch et al., 1995a, 1995b) are responsible for neurotrophin binding, whereas other similar studies suggest that the IgG–C2 subdomain is most important (Perez et al., 1995; Urfer et al., 1995). Recent cellular and in vivo data support the importance of the LRM repeats (MacDonald & Meakin, 1996; Ninkina et al., 1997). Thus, the extracellular domain of the Trk receptor could contribute two separate binding regions for neurotrophins, which could be acting separately or together, depending on the cellular context.

The extracellular domain of TrkA has now been overexpressed in Sf21 insect cells as part of an effort using purified components to understand the complicated interplay between NGF and its receptors. Biophysical and biochemical properties of homogeneous TrkA receptor extracellular domain (TrkA-RED) have been determined, including real-time binding kinetics with Biosensor instrumentation. We demonstrate that the recombinant TrkA-RED was properly processed as a fully folded, glycosylated polypeptide and behaves as a "low-affinity" NGF receptor when the kinetics are

analyzed with Biosensor technology. Formation of a complex of the NGF dimer with two TrkA-RED molecules and a resultant conformational change are also demonstrated.

Results

Expression and purification of recombinant TrkA-RED

TrkA-RED was expressed at a level of 5 mg/L of Excell 401 serum-free medium when assessed in a Western blot using polyclonal anti-TrkA antibodies. The apparent molecular mass observed on 12% reducing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was about 70 kDa, while the molecular mass predicted from the amino acid composition of the translation product for the cDNA corresponding to the extracellular domain would be 41 kDa. The discrepancy must arise from the carbohydrate content acquired by glycosylation when the protein is expressed in Sf21 cells. There are 13 potential N-glycosylation sites that may be glycosylated when expressed in a eukaryotic expression system (Martin-Zanca et al., 1989; Woo et al., 1995). The presence of carbohydrate was confirmed by subsequent experiments (see below). TrkA-RED was tightly bound to the ConA-Sepharose column. This affinity column is useful for separating proteins that contain α -D-mannopyranosyl, α -D-glucopyranosyl, and sterically related residues (Goldstein et al., 1965). As expected, TrkA-RED was eluted efficiently with α -D-mannoside dissolved in buffer. Table 1 shows that 2 mg of pure TrkA-RED (Fig. 1) were isolated from a liter of Excell 401 serum-free medium.

The NH₂-terminal sequencing identified the sequence (APCP DACCPH...) as that of predicted mature TrkA except for a missing initial alanine residue (Martin-Zanca et al., 1989). Sequencing of the baculoviral DNA showed the presence of the codon for the preceding alanine residue. The reason that the first alanine residue was deleted is not clear, but improper proteolytic processing could have happened during the expression. Analysis of the total amino acid composition together with the results of the NH₂-terminal sequencing indicates that the recombinant TrkA-RED is authentic. The high carbohydrate content of TrkA-RED did not interfere with the quantitation of the amino acid composition. These results indicate that the Sf21 cell was a reliable protein expression system for authentic TrkA-RED. The purity of the final preparation was greater than 95%, as judged by Coomassie staining (Fig. 1).

Table 1. Purification of TrkA-RED (from 1.2 L of cell supernatant)

Purification step	Total protein ^a (mg)	TrkA-RED ^b (mg)	Purity ^c (%)	Overall yield (%)
1. Supernatant (Excell 401)	1,000	12	1.2	100
2. (NH ₄) ₂ SO ₄ precipitation	157	6.3	4	53
3. DE-52 anion exchange column	45	3.8	8.5	32
4. Sephacryl S-200 gel filtration	17.1	3.6	21	30
5. ConA sepharose column	6.3	3.3	48	28
6. Hydroxyapatite column	2	2	100	17

^aDetermined with the Bradford assay.

^bDetermined by Western blotting.

^cImmunodetected TrkA-RED divided by total protein.

Fig. 1. SDS PAGE of purified TrkA-RED. The purified TrkA-RED was separated on a 12% SDS polyacrylamide gel. Lane 1: low-range prestained protein molecular weight markers from Bio-Rad. Lanes 2–5: 12, 6, 3, and 1.5 μ g of TrkA-RED stained with Coomassie Blue R250.

Determination of molecular weight and degree of association

Trk families of protein tyrosine kinase receptors are heavily glycosylated on the extracellular region as shown with several different neurons as well as a neuronal phenotype PC12 cells (Barbacid, 1994). The exact structure of the carbohydrate for each Trk receptor has not been investigated. The intact TrkA in PC12 cells and in dorsal root ganglia neurons migrates with an apparent molecular mass of 140 kDa on a 7% SDS-PAGE, suggesting that approximately 40% of the apparent molecular weight is contributed by carbohydrate. TrkA-RED expressed in Sf21 insect cells migrated with apparent molecular mass of 70 kDa (Fig. 2A), which is more than 70% larger than the predicted one. This difference suggests that the recombinant TrkA-RED is well glycosylated in insect cells as with the extracellular domain of the intact TrkA in PC12 cells.

The molecular mass of TrkA-RED under native conditions was estimated either from the elution position on an FPLC gel filtration column (Fig. 2B) or from flow measurements on a light-scattering instrument in tandem with the gel filtration column (Fig. 2C). A calibration curve was determined from five different protein standards on a Superose 12 FPLC gel filtration column that has a resolution range of 5,000 to 250,000 Da. TrkA-RED displayed an elution position (V_e/V_o) corresponding to a molecular mass of 116 kDa in these measurements (Fig. 2B).

The PD2000 light-scattering instrument (Precision Detectors, Inc.) is arranged to collect refractive index, low-angle and right-angle light-scattering data simultaneously and to calculate molecular weight distributions, molecular sizes, and species concentrations.³ The molecular mass of a macromolecular species is calculated as a function of the intensity of scattered light and refractive index (Tanford, 1961; Katima & Quintana, 1989). The major peak (>90%) was estimated to have a weight-average molecular mass of 55 to 60 kDa, with different preparations in separate experiments. The ratio, 1.01, of the weight average (M_w) to the number average (M_n) mass indicated solution homogeneity of the species at this elution position. Two additional minor species were also detected by light scattering corresponding to about 144 and 246 kDa. These minor

species could be dimers or higher oligomers of TrkA-RED. Previous whole-cell crosslinking studies have observed oligomeric receptor species when NGF was bound to full-length TrkA that had been overexpressed in fibroblast cells (Jing et al., 1992).

Presence of carbohydrate on the TrkA-RED surface was demonstrated by treatment with N-glycosidase F (PNGase F, peptide-N⁴-(acetyl- β -glucosaminyl)-asparagineamidase, EC3.2.2.18), which hydrolyzes all types of N-glycan chains from glycoproteins unless they carry an α -1,3-linked core fucose residue found in insect and plant glycoproteins. This enzymatic treatment should cause a change in molecular weight, structure, or properties of the polypeptide. Two time courses were set up for digestion by PNGase F in the presence and absence of SDS and 2-mercaptoethanol (Fig. 3A,B). Digestion in the denaturation conditions shifted the molecular mass from 70 to 50 kDa (~40% decrease) within 24 h when incubated at 37 °C, whereas deglycosylation without those reagents was much slower. After treatment, carbohydrate was visualized by staining with Schiff's base after periodate oxidation (Gerard, 1990). Treatment of TrkA-RED with PNGase F for 24 h, either with or without SDS, decreased staining for carbohydrate below detection limits (data not shown). The value of 50 kDa is slightly larger than that of 41 kDa from the predicted amino acid sequence, suggesting that some carbohydrate was not removed by the N-glycosidases.

Inhibition of neurite outgrowth in PC12 cells by TrkA-RED

Signaling by NGF and related family members is initiated by forming the ligand-receptor complex between the neurotrophin and a neurotrophin-specific Trk receptor (Kaplan & Stephens, 1994). This type of signaling can be interrupted by introducing a competitor molecule, either for the neurotrophin or for the Trk receptor. PC12 cell bioassays were utilized in which the TrkA-RED concentration was varied from 10 pM to 100 nM. The IC₅₀ for inhibiting neurite outgrowth was estimated to be ~1 nM, which corresponds to a "low" affinity for NGF binding. An excess of TrkA-RED (100 nM) introduced in the defined PC12 bioassay inhibited approximately 90% of the neurite outgrowth when 0.2 nM NGF (5.3 ng/mL) was used.

Formation of NGF-TrkA-RED complex

The formation of the TrkA-RED-NGF complex was demonstrated by gel filtration chromatography with Superose 12 FPLC column. An NGF dimer molecule could potentially interact with one or two molecules of TrkA-RED; therefore, the stoichiometry was carefully monitored. NGF was incubated at pH 7.4 with TrkA-RED for 1 h at 37 °C, the mixture was then separated by a Superose 12 FPLC gel filtration column, and the molecular mass estimated by both light-scattering and elution position. The native TrkA-RED resulted in one peak with a weight average molecular mass of 55 kDa. A mixture of NGF and TrkA-RED at a molar ratio of 2 (NGF dimer to TrkA-RED monomer) gave a homogenous peak ($M_w/M_n = 1.01$) of 134 kDa, as well as a small peak for free NGF (Fig. 4). Molecular mass estimations from the calibrated elution position were in agreement in the shift of the molecular mass position, but with higher values consistent with that described above for TrkA-RED (Fig. 2B,C). An experiment at lower concentrations (1.9 μ M NGF and 1.4 μ M TrkA-RED) and a molar ratio of 1.3 produced three peaks corresponding to the complex, free TrkA-RED, and free NGF. This result demonstrates the dynamic equilibrium existing under these conditions with a reason-

³Manufacturer's Manual, 1994. A macromolecular characterization system using a light-scattering intensity detector and a concentration detector. Amherst, Massachusetts: Precision Detectors, Inc.

Fig. 2. Determination of the molecular weight of TrkA-RED by SDS-PAGE, Superose 12 FPLC gel filtration, and light scattering. **A:** The SDS-PAGE (Fig. 1) of TrkA-RED (open circle) was used to calculate the molecular mass. **B:** TrkA-RED was run on a Superose 12 FPLC gel filtration column equilibrated with PBS, pH 7.2 (7.5×30 mm, Pharmacia). The elution volume of TrkA-RED was compared with those of the protein calibration standards. **C:** PD2000 light-scattering instrument (Precision Detectors, Inc.) tandemly attached to Superose 12 FPLC column and equilibrated with PBS, pH 7.2, was used to determine the molecular mass of TrkA-RED. Note that the light-scattering line accentuates the contribution of the earlier eluting peaks because of their greater size and contribution to the signal.

able dissociation constant. Due to the inability to account for dilution during the gel filtration and the perturbation during separation, no attempt was made to determine the K_d from these experiments.

The peak samples from the Superose 12 column were separated in SDS-PAGE, stained with Coomassie Blue R250, and quantitated against standard samples to confirm the stoichiometry of the complex. The 134 kDa peak from the mixture of NGF and the native TrkA-RED displayed two electrophoretic bands, corresponding to NGF and TrkA-RED, with a mean ratio of 1.6 TrkA-RED monomers to each NGF dimer, with a variation in the ratio of 1.2 to 2.5 when measured in individual tubes within the peak fraction (data not shown).

The deglycosylated TrkA-RED aggregated into a higher molecular weight position on the Superose 12 column, yielding a broad peak at around 600 kDa. Incubation of NGF with the deglycosylated TrkA-RED decreased the height of the 600 kDa peak to almost none (data not shown). These results suggest that carbohydrate on the Trk receptors could play a role in maintaining the structure and preventing aggregation of TrkA-RED.

The presence of the TrkA-RED-NGF complex was also confirmed by affinity labeling with 125 I-NGF. Affinity labeling was performed with disuccinimidyl suberate, an effective crosslinking agent for Trk (Hosang & Shooter, 1985; Kaplan et al., 1991; Meakin & Shooter, 1991). Autoradiography after SDS-PAGE displayed a

Fig. 3. Time course of deglycosylation of TrkA-RED. TrkA-RED (25 μ g) was incubated in 0.1 M sodium phosphate, pH 8.5, with 0.5 units of N-glycosidase F (Boehringer-Mannheim) in the presence (A) or absence (B) of 0.1% SDS and 1% 2-mercaptoethanol. Aliquots were withdrawn at 2, 4, and 24 h after incubation and immediately boiled to make the SDS-PAGE samples. Proteins were separated on a 12% SDS-PAGE and stained with Coomassie blue R250. Lane 1: low-range prestained protein molecular weight markers (Bio-Rad). Lanes 2–5: 10 μ g of TrkA-RED at 0, 2, 4, and 24 h at 37°C in the absence of N-glycosidase F. Lanes 6–8: 10 μ g of TrkA-RED treated with N-glycosidase F for 2, 4, and 24 h at 37°C.

Fig. 4. FPLC gel filtration chromatography of the NGF-TrkA-RED complex. NGF (152 μ g) was incubated with 200 μ g of TrkA-RED in 200 μ L of PBS, pH 7.4, for 1 h at 37°C and the mixture was separated on a Superose 12 FPLC gel filtration column (Pharmacia). Closed squares: TrkA-RED (14 μ M monomer final) plus NGF (28 μ M dimer final). Open circles: TrkA-RED only (28 μ M).

major band at 85 kDa (Fig. 5), while some experiments indicated a minor band at 160 kDa representative of a TrkA-RED dimer (data not shown). Both bands disappeared when a 1,000-fold excess of unlabeled NGF was used in the competition assay, confirming the presence of specific complex species. An additional band at about 26 kDa was observed (Fig. 5A, lanes 1 and 2) at the higher unlabeled NGF concentrations, indicative of crosslinked

Fig. 5. Affinity crosslinking of 125 I-NGF to TrkA-RED. 180 nM of TrkA-RED in PBS, pH 7.4, containing 0.1% BSA was incubated with 1 (A) or 10 (B) nM 125 I-NGF (specific radioactivity, 100 cpm/pg) for 45 min at 37°C in the presence or absence of an unlabeled competitor. Bound 125 I-NGF was crosslinked to TrkA-RED for 30 min at room temperature using DSS at a final concentration of 150 μ M and separated on 7% SDS-PAGE. Radioactive bands were visualized by autoradiography and intensities were measured with a Molecular Image System (Bio-Rad). The arrowhead indicates the position of the 85 kDa NGF-TrkA-RED complex. A: Lanes 1–9: concentrations of unlabeled NGF, as indicated. B: Lane 1: 1 μ M NGF; Lanes 2 and 3: 1 and 0.1 μ M BDNF; Lane 4: 1 μ M NT3; Lane 5: 1 μ M NT4/5; Lane 6: no TrkA-RED and no unlabeled competitor; Lane 7: no unlabeled competitor. C: Quantitation of the dose-response data from A. The apparent EC_{50} was 184 nM.

NGF dimer as previously reported (Timm et al., 1994). In contrast, competition with 1 μ M BDNF, NT-3, or NT-4/5 did not noticeably reduce the TrkA-RED band (Fig. 5B). Similar competition assays were performed with a dilution series of unlabeled NGF to quantitate the binding of NGF to TrkA-RED (Fig. 5A,C). Data were collected and quantitated with a Molecular Image System (Bio-Rad) to give an apparent EC_{50} of 184 nM. This apparent value is high because of the experimental conditions of a very high TrkA-RED concentration of 180 nM that is necessary for adequate sensitivity of the crosslinked band (Timm et al., 1994). After appropriate corrections for a stoichiometric titration of a tight-binding competitor (Williams & Morrison, 1979), the data are consistent with a K_d in the range of 1 to 10 nM, which is in accord with our other data and with previous results observed with intact TrkA ectopically expressed on Sf21 insect cells (Woo et al., 1995).

Biosensor analysis of the NGF-TrkA-RED interaction using surface plasmon resonance

One of the emerging techniques to measure the interaction between ligand and its cognate receptor is to use an optical surface plasmon resonance detection for monitoring the interaction in real time (Jonsson et al., 1991). In this analysis either ligand or receptor can be immobilized onto an appropriate sensor chip. The amount of either NGF or TrkA-RED immobilized to the carboxymethylated CM5 sensor chip using N-ethyl-N'-(3-diethylaminopropyl) carbodiimide/N-hydroxysuccinimide (EDC/NHS) chemistry was estimated from the surface plasmon resonance signal. One thousand response units (RU) is equivalent to a change of about 1 ng/mm² in surface protein concentration.⁴ The surface was best regenerated with the phosphoric acid-glycine regeneration buffer. Real-time kinetic measurements were performed with several different immobilized TrkA-RED chips and various NGF concentrations in HEPES-buffered saline (HBS). A typical experiment is shown in Figure 6.

Whether mass transport affects the apparent on-rate of the reaction was tested with chips of different ligand density. A low density chip with 400 RU of TrkA-RED immobilized displayed similar on-rates as for a chip with 700 RU immobilized, suggesting that any mass transport effect was minimal in this range of ligand density. One experiment with a higher density (1000 RU) chip, however, recorded somewhat slower on-rates (data not shown), suggesting that mass transport may be a limiting factor under these conditions. A limit coefficient⁴ for the mass transport ($k_a[B]/k_m$) of NGF with the TrkA-RED high density chip could be more than 0.5. The correlation that a mass transport-limited association is followed by a mass transport-limited dissociation was tested by injecting free TrkA-RED in the buffer used during the dissociation phase. The free TrkA-RED will compete with the immobilized TrkA-RED for binding of the released NGF and prevent its re-binding onto the chip. Addition of free TrkA-RED did not increase the apparent off-rate of bound NGF from the 700 RU chip, suggesting that the rebinding phenomenon did not limit the dissociation. Additionally, measurement of the off-rates at low (10 μ L/min) or high (40 μ L/min) flow rates gave identical results. We conclude that the data from the 400–700 RU chips (Table 2) are an accurate determination of the relative kinetic rates of the system.

⁴Manufacturer's Manual, 1994. *BIAtechnology and BIAapplications Handbook*. Uppsala: Pharmacia Biosensor AB.

Table 2. Kinetic constants of NGF binding to TrkA-RED surface

	$k_{off} \pm SD$ [s ⁻¹ ($\times 10^3$)]	$k_{on} \pm SD$ [M ⁻¹ s ⁻¹ ($\times 10^{-5}$)]	$K_d(eq)$ (nM)
Exp 1 ^a	1.34 $\pm$ 0.18	6.71 $\pm$ 0.45	2.0
Exp 2 ^a	1.61 $\pm$ 0.29	5.04 $\pm$ 1.08	3.2
Exp 3 ^a	1.97 $\pm$ 0.53	5.09 $\pm$ 1.16	3.9
Mean ^b	1.64 $\pm$ 0.32 (19%)	5.61 $\pm$ 0.95 (17%)	2.92 $\pm$ 0.75 (26%)

^aMean and standard deviation (SD) calculated from 5, 3, or 6 sensorgram curves in Experiments 1, 2, and 3, respectively.

^bMean of the k_{off} and k_{on} values from the three experiments with SD propagated appropriately. The mean $K_d(eq) \pm$ propagated SD was calculated from the ratio of the mean rate constants.

The rate constants for NGF interaction with TrkA-RED were determined from 14 sensorgram curves in three separate experiments on two chips (400 and 700 RU). The average values of the on and off rate constants were $5.61 (\pm 0.95) \times 10^5$ M⁻¹s⁻¹ and $1.64 (\pm 0.32) \times 10^{-3}$ s⁻¹, respectively (Table 2). The ratio of kinetic constants (k_{off}/k_{on}) for NGF binding to TrkA-RED in this measurement gave rise to an average equilibrium dissociation constant, K_d , of $2.92 (\pm 0.75) \times 10^{-9}$ M, similar to the values previously measured with intact TrkA on cell surfaces (Hempstead et al., 1991; Mahadeo et al., 1994; Woo et al., 1995; Woo & Neet, 1996).

The binding affinity can also be assessed from the steady-state plateau values⁴ of each curve (Hammacher et al., 1996). Fitting a binding isotherm to these values and visualizing the results in the form of a Scatchard plot ($R_{eq}/concentration$ versus R_{eq}) for various

Fig. 6. Surface plasmon resonance sensorgram for association and dissociation of NGF with immobilized TrkA-RED on the BialiteTM Biosensor. The raw data curves show the response units (RU) as a function of time (s) at 200, 100, 80, 50, 30, and 15 nM NGF analyte (top to bottom). The flow rate was 10 μ L/min, data collection rate was 10 points/s, and the temperature was 25 $^{\circ}$ C in 10 mM HEPES, pH 7.4, 150 mM NaCl, 3.4 mM EDTA, 0.005% (v/v) Surfactant P20. The average rate constants obtained in this particular experiment were $k_{on} = 6.28 (\pm 1.55) \times 10^5$ M⁻¹ s⁻¹ and $k_{off} = 2.39 (\pm 0.42) \times 10^{-3}$ s⁻¹.

NGF concentrations produced a value of $K_d = 6.1 (\pm 1.4)$ nM (data not shown). This value from equilibrium conditions agrees within a factor of about 2 with the value from the kinetic constants, 2.9 nM, satisfying one of the conditions suggested for testing biosensor data (Shuck & Minton, 1996).

The converse experiment with immobilized NGF and TrkA-RED as the analyte produced more complex kinetics with an overall lower affinity than the data described above with immobilized TrkA-RED. The immobilization chemistry, which used activated carboxyl groups on the dextran matrix and lysine residues in NGF, could provide an unusual topology for subsequent receptor binding either by steric hindrance or a global conformational change. Previously, some lysine residues in NGF were shown to have no influence on TrkA receptor binding (Ibanez et al., 1992; Bradshaw et al., 1994; Ibanez, 1994). However, unfavorable physical immobilization of the NGF dimer could at least partially block the free access of TrkA-RED toward the proposed TrkA binding sites located on the dimeric NGF surface. The global conformation of the immobilized NGF seemed unchanged, because the N60 monoclonal antibody still bound with high affinity (data not shown).

Circular dichroism (CD) analysis of the NGF-TrkA-RED interaction

The CD spectra of both NGF and TrkA-RED are reported in Figure 7A. As previously reported, the spectra of NGF consists primarily of contributions from β -strand and random coil structure (Radziejewski et al., 1992; Timm & Neet, 1992; Narhi et al., 1993). The spectra of TrkA-RED has a characteristic contribution from α -helical structure in the 215–230 nm region and from β -structure in the 195–205 nm region. Analysis of this spectra by the LINCOMB and CCA programs developed by Fasman and colleagues (Perczel et al., 1992) indicated that the structure could be described by 66 to 68% β -sheet, 7 to 16% α -helix, and 10 to 15% random coil. The high amount of β -structure is consistent with two IgG-like domains in TrkA-RED, predicted from the sequence. The conformation of this extracellular domain is distinct from that of p75LANR, which is mainly random coil (Timm et al., 1992), but is similar to that of other receptor tyrosine kinases such as the epidermal growth factor (EGF) receptor and the insulin receptor (Greenfield et al., 1989; Schaefer et al., 1992).

We have previously shown a small change in conformation by CD measurements when each of the neurotrophins interacts with the common low-affinity receptor, p75LANR (Timm et al., 1992, 1994). A similar experiment was conducted to determine if a conformational change occurred when TrkA-RED bound NGF. NGF and TrkA-RED were mixed to form the complex, and the CD spectra was compared to that of a simple summation of spectra from the individual components. If no change in secondary structure occurred, the mixed and calculated spectra should be identical. The result (Fig. 7B) clearly showed a small but statistically significant difference spectrum. This change was reproducible, specific for this interaction, and not due to small differences in estimation of the concentrations of the components. The magnitude of the difference spectrum is similar to that observed with formation of the EGF complex with the EGF receptor extracellular domain (Greenfield et al., 1989).

Discussion

This study has described the preparation and characterization of homogeneous recombinant TrkA-RED from ectopic expression in

Fig. 7. CD spectra analysis of the NGF and TrkA-RED interaction. **A:** CD spectra in mean residue ellipticity ($\text{deg} \cdot \text{cm}^2 / \text{mol} \times 10^{-3}$) of 3 μM NGF (NGF), 0.9 μM TrkA-RED (TrkA-RED), a 1:1 mixture of the two samples (MIX), and the calculated CD spectra from the digital averaging of the spectra of the two components measured separately (CALC). **B:** Difference spectra obtained by subtracting the NGF/TrkA-RED calculated spectra from the experimental spectra of NGF/TrkA-RED mixtures and averaged over three separate experiments; a smoothed curve is drawn through the data. All spectra were recorded in a 2-mm path cell in pH 7, 10 mM sodium phosphate buffer.

Sf21 insect cells. Binding of NGF to TrkA-RED immobilized on the biosensor generated monophasic association and dissociation rates with an appropriate K_d of about 2 nM. These studies have allowed us to make several significant observations about the interactions between NGF and the extracellular domain of TrkA.

TrkA-RED is a heavily glycosylated protein with a molecular mass of 70 kDa as determined by SDS-PAGE. The native molecular mass of the TrkA-RED monomer was estimated to be 116 kDa by calibration of a Superose 12 column and 55 kDa by calculation of the light-scattering data from the same peak. We believe that the high value (116 kDa) from the Superose column is due to hydrodynamic properties and not due to oligomerization of free TrkA-RED. The protein standards used for calibration of the Superose are not glycoproteins, as is TrkA-RED, leading to poor assumptions about the relative Stokes radius. The values of 55 and 70 kDa from the light scattering and SDS gels, respectively, are in reasonable agreement, considering the assumptions involved in both values about the contribution of the carbohydrate to the estimated molecular weight.

Evidence for dimerization of the TrkA extracellular domain in the presence of NGF was provided by two observations. The mo-

molecular mass, as determined by light scattering, of the most prominent peak on gel filtration moved from 55 to 134 kDa, consistent with an NGF dimer-TrkA-RED dimer complex ($55 + 55 + 26 = 136$). Simple addition of an NGF dimer of 26 kDa to the 55 kDa species would not give a molecular mass this high. Similarly, calculations of the molecular mass from the elution position of these peaks also resulted in an increase from 110 to ~ 200 kDa, substantially more than the simple addition of one NGF dimer to the size of the TrkA-RED. SDS-PAGE and Coomassie staining of the eluted peaks confirmed that the stoichiometry of these species was about 2:1 TrkA-REDs per NGF dimer. Affinity labeling also gave some evidence for a 2:1 complex of TrkA-RED to NGF dimer. Although the absolute amounts of product in crosslinking experiments are not reliable due to inefficiency of the crosslinking, the presence of small amounts of the 160 kDa band, in addition to the 85 kDa band of the monomer (70 plus 13 kDa), indicates that some crosslinking of two TrkA-RED molecules to one NGF dimer occurred. Thus, dimerization of TrkA-RED is induced by NGF, analogous to that found for other receptor tyrosine kinases such as the EGF receptor (Lax et al., 1991).

Several pieces of information suggest that the second association step between NGF and TrkA-RED may be weaker than the first. Only a small proportion of TrkA-RED is in the DSS cross-linked dimer, relatively high concentrations of NGF were required to shift all of the TrkA-RED into the dimer peak on gel filtration, and the ratio determined from SDS-PAGE of the Superose peak was slightly lower than the expected value of 2. Such a relationship between first and second binding sites has also been reported for growth hormone (Wells, 1996), erythropoietin (Philo et al., 1996), and other polypeptide ligands. A weaker second site binding is consistent with the observation of only a single binding phase and a 1:1 (TrkA-RED monomer:NGF dimer) stoichiometry under the conditions of the biosensor experiments. Evidence for a 2:1 stoichiometry with a strong and a weak binding site has been presented for TrkB and Trk C with BDNF and NT-3 utilizing equilibrium sedimentation methods (Philo et al., 1994). However, in those studies, the equilibrium dissociation constant calculated for the complex was two to three orders of magnitude higher than ours with TrkA and therefore of problematic physiological consequence. At this time, we are uncertain whether the differences between these studies are due to differences in the Trk preparations or in the methodology utilized; however, the values of K_d in this report are consistent with physiological values measured with intact cells (Hempstead et al., 1991; Mahadeo et al., 1994; Woo et al., 1995; Woo & Neet, 1996).

The dimerization of the extracellular domain of TrkA induced by NGF occurs concomitantly with a conformational change in one or both components as observed by CD measurements. The secondary structure is less ordered in the complex than as free components in solution with decreases in the β strand/random coil region (195–210 nm) as well as the α -helix position (220–230 nm). The magnitude and direction of change are similar to that observed for other receptor tyrosine kinase extracellular domains (Greenfield et al., 1989; Schaefer et al., 1992). These effects of dimerization and conformational change may lead to initiation of the signal cascade by transautophosphorylation of the cytosolic catalytic domains or may be incidental to such activation.

The on- and off-rates of NGF binding to TrkA-RED determined in the biosensor would be classified "slow" in the NGF system. The association rate was close to the reported value measured at 4 °C (Mahadeo et al., 1994). The rate is 20 times slower than that

of the high-affinity binding sites when both p75 LANR and TrkA are expressed together in PC12 cells, and 10 times slower than that for p75LANR alone (Woodruff & Neet, 1986; Meakin & Shooter, 1992; Mahadeo et al., 1994; Chao & Hempstead, 1995). The slow on-rate for NGF binding to TrkA-RED in this measurement gave rise to a "low" equilibrium affinity for NGF with a K_d of 2.0 nM. This value was confirmed by a Scatchard analysis of the plateau data, by inhibition of the bioactivity of NGF, and by the concentration dependence of chemical crosslinking with DSS. These data suggest that the structure and function of the recombinant TrkA-RED are comparable to the extracellular domain of the intact TrkA receptor in neuronal cells. Thus, this polypeptide and its recombinant mutant forms may be used as a biochemical tool to characterize the interaction of NGF and TrkA.

Different subdomains of the TrkA molecule have been reported to have different binding affinities for NGF (Perez et al., 1995; Urfer et al., 1995; Windisch et al., 1995b). When a TrkC IgG-like subdomain located adjacent to the transmembrane domain was exchanged for the homologous sequence from TrkB or TrkA, the resultant chimera demonstrated binding to BDNF or NGF, respectively (Urfer et al., 1995). This second IgG-like subdomain, alone, retained an affinity for neurotrophins similar to the full-length receptor and also showed neurotrophin dependent activation when expressed on NIH 3T3 cells in fusion with the kinase domain (Urfer et al., 1995). However, the high affinity binding (20–150 pM) observed in these experiments may be due to avidity effects of the bivalency of the immuno-adhesion fusion molecule. Similar experiments with subdomain-swapped TrkA chimeras expressed in 293 cells, but without using the immuno-adhesion construct, also implicated the two IgG-like subdomains as the region of NGF binding but reported a higher K_d of about 1 nM (Perez et al., 1995). On the other hand, a single leucine-rich motif (LRM) of 24 amino acids was reported to bind NGF selectively with nM affinity (Windisch et al., 1995b), and the binding kinetics were equivalent to that observed for the entire recombinant extracellular domain. Whether the LRM or the IgG-like subdomain is the main contributor to neurotrophin binding is still open to question.

Binding sites of high affinity in the range of 5–10 pM have been described for some cell lines overexpressing TrkA receptor alone (Klein et al., 1991; Jing et al., 1992). No evidence for a dissociation constant less than 1 nM was found in our study. The lack of the formation of dimeric or oligomeric TrkA-RED when immobilized on the sensor chip may prevent formation of a high-affinity homodimeric receptor site (Jing et al., 1992). Another possibility is that the proximal presence of both p75LANR and TrkA-RED is required for the formation of the high-affinity heterodimeric receptor sites for neurotrophins in general (Chao & Hempstead, 1995). The immobilized biosensor system reported here will allow the investigation of some of these possibilities in a defined, controlled system with functional, purified proteins.

Materials and methods

Reagents

β NGF was purified from the submaxillary gland of adult male Swiss-Webster mice through the 7S NGF form by using Sephacryl S200, CM52, and monoS FPLC as described previously (Woodruff & Neet, 1986; Luo & Neet, 1992). The mature NGF polypeptide was more than 95% intact, as determined by reducing SDS-PAGE and nonequilibrium isoelectric focusing (Longo et al., 1989) and

had full biological activity in the PC12 bioassay. Monoclonal anti-NGF antibody N60 was produced as described previously (Neet et al., 1987). Polyclonal anti-NGF antibody was from CRC, Inc. Polyclonal anti-TrkA antibodies and TrkA-RED baculovirus were generously donated by Dr. Kaplan at the Montreal Neurological Institute. Horseradish peroxidase conjugate of secondary antibodies to rabbit was from Organon Technika Co., Durham, NC. CM5 sensor chips, amine coupling reagents, and HBS buffer were supplied by Pharmacia Biosensor (Biacore, Piscataway, NJ).

Construction and expression of TrkA-RED Baculovirus

cDNA of the human TrkA-RED was subcloned in the 9.3 kb pAcC4 Baculovirus vector at the Nco I and EcoR I sites [(Stephens et al., 1994); personal communication from Dr. D. Kaplan]. The EcoR I site was created by polymerase chain reaction at the 3' end of the cDNA. Positive baculovirus for TrkA-RED was screened and amplified to 10^8 pfu/mL in serum-containing media. Overexpression of TrkA-RED in Sf21 insect cells in Excell 401 serum-free media (JRH Biosciences, Lenexa, Kansas) was handled as described previously (Luo & Neet, 1992; Woo et al., 1995).

Purification of TrkA-RED

(a) Granular ammonium sulfate was added with gentle stirring to make the insect cell supernatant 80% saturated at 4°C. The solution was centrifuged for 30 min at 10,000 rpm in a Beckman JA-10 rotor. Pellets were dissolved in a minimum volume of 25 mM Tris·HCl, pH 8, and dialyzed for 24 h. (b) A DE52 anion exchange chromatographic column was loaded with the dialyzed sample at 5 mg protein/mL DE52 bed volume. TrkA-RED was eluted at 100 mM NaCl in the equilibrium buffer. The fractions containing TrkA-RED were pooled and concentrated to 3 mL using an Amicon PM30 membrane, and the concentrate was dialyzed against 200 mM NaCl in 25 mM Tris·HCl, pH 8. (c) Sephacryl S-200 gel filtration chromatography (2.5×100 cm, D $\times$ L) was used to further purify the protein. The dialysate was loaded and eluted at a flow rate of 45 mL/h and 200 fractions of 3 mL were collected. Fractions containing TrkA-RED were pooled and concentrated to 10 mL. (d) Ten milliliters of ConA-Sepharose (Pharmacia, Uppsala, Sweden) was equilibrated with 25 mM Tris·HCl, pH 8, containing 200 mM NaCl, 1 mM CaCl₂, and 1 mM MnCl₂, and the sample was loaded. Unbound proteins were washed off with successive applications of the column equilibrium buffer and then 50 mM α -D-methylmannoside in the same buffer. TrkA-RED was eluted with 500 mM α -D-methylmannoside and the pool concentrated to 10 mL. The concentrate was dialyzed against 1 mM potassium phosphate, pH 6.8, overnight. (e) Hydroxyapatite affinity chromatography was performed by suspending dry powder of HTP (Bio-Rad) in 1 mM potassium phosphate, pH 6.8, and packing into a 10-mL disposable column. Loaded proteins were eluted in a batch mode using increasing concentrations of potassium phosphate, pH 6.8. Pure TrkA-RED was eluted at 50 mM potassium phosphate. Table 1 shows the yield and activity after each step.

PC 12 bioassay

PC12 cells were maintained in serum-containing media and defined medium bioassays were performed as described previously (Reinhold & Neet, 1989). In the inhibition assays, the NGF con-

centration was fixed at 0.2 nM (5.3 ng/mL medium), whereas the TrkA-RED concentration was serially diluted from 100 to 0.1 nM. The number of neurite-bearing cells was counted 48 h after incubation.

Analysis of glycosylation

TrkA-RED (25 μ g) in 0.1 M sodium phosphate, pH 8.5, was incubated with 0.5 units of N-glycosidase F (BMB) at 37°C. The carbohydrate content remaining in the samples was monitored by carbohydrate staining with the Schiff's base after periodate oxidation (Gerard, 1990) and protein staining in SDS-PAGE.

Analysis of NH₂-terminal sequence and amino acid composition

NH₂-terminal sequence was analyzed by on-line identification of the phenylthiohydantoin derivatives on an ABI 470A protein sequencer. Protein samples were hydrolyzed in 6 N HCl containing 0.1% phenol at 150°C for 1 h and the amino acid composition was determined as the derivatives with phenylisothiocyanate as described previously (Woo & Neet, 1996).

Determination of molecular weight

Molecular weight was determined by SDS-PAGE, Superose 12 FPLC gel filtration, and light scattering. TrkA-RED was electrophoresed on a 12% reducing SDS-polyacrylamide gel with the prestained protein standards (Bio-Rad) and their migration distances were compared. A Superose gel filtration column was calibrated with a set of protein calibration standards (Sigma) and the elution volume was compared with those of standard proteins. Molecular weight was also determined on a Precision Detectors PD2000 light-scattering instrument tandemly connected with a Waters 410 Differential Refractometer. TrkA-RED (0.2 mL) (1 mg/mL in buffer) was loaded on a Superose 12 FPLC column (7.5×300 mm), the change in refractive index and right-angle light scattering were recorded with a PC computer, and the data were analyzed by the software supplied by Precision Detector, Inc.

Crosslinking of NGF to TrkA-RED

TrkA-RED (180 nM in Dulbecco's phosphate buffered saline, pH 7.4, supplemented with 0.1% BSA) was incubated with ¹²⁵I-NGF (specific activity of 100 cpm/pg protein) for 45 min at 37°C either with unlabeled NGF, BDNF, NT3, or NT4/5. DSS in DMSO was added to a final concentration of 150 μ M and incubated for 30 min at room temperature to crosslink NGF to TrkA-RED. Samples were boiled in sample buffer and separated on a 12% SDS-PAGE and visualized either by autoradiography (Timm et al., 1992) or using a GS-363 Molecular Imager System (Bio-Rad). The relative band intensity was fit to a simple competition curve by a nonlinear least-squares fitting program to obtain an apparent EC₅₀ (Fig. 5C). Because the concentration of TrkA-RED is more than a 100-fold higher than the true K_d , this situation is analogous to a tight-binding inhibitor (Williams & Morrison, 1979). The apparent EC₅₀ must be corrected by subtracting half of the TrkA-RED concentration and by the term $([^{125}\text{I-NGF}]/K_d(\text{NGF}) - 1)$ to obtain the true K_i for the competitor, as given in Equation 7 of Williams and Morrison (1979).

Kinetic measurements of NGF and TrkA-RED interactions

Either mouse β NGF or TrkA-RED (5 μ g/mL in 10 mM sodium acetate coupling buffer, pH 4.8) was immobilized to a CM5 sensor chip using EDC/NHS coupling chemistry. All reagents were introduced over the sensor chip in 10 mM HEPES, pH 7.4, 0.15 M NaCl, 3.4 mM EDTA, and 0.005% Tween 20 (HBS buffer) at a flow rate of 5 or 10 μ L/min. Binding isotherms were determined at 25°C. The sensor surface was regenerated between assays by treatment with either 10 mM glycine-HCl, pH 2, or a 1:1 mixture of 100 mM phosphoric acid and 10 mM glycine-HCl, pH 2. The sensorgram data from real-time biomolecular interaction analysis were evaluated with the BIA evaluation software package (Pharmacia Biacore).

Circular dichroism (CD)

CD spectra were recorded on a Jasco 710 spectropolarimeter with computer control and data acquisition at room temperature. Protein samples including appropriate controls were measured in a quartz cuvette with a 2-mm path length at 50 μ g/mL protein concentration in 10 mM potassium phosphate, pH 7. Spectra were recorded as an average of 6 to 12 scans from 260 to 185 nm.

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