Binding of GDNF and Neurturin to Human GDNF Family Receptor α 1 and 2

INFLUENCE OF cRET AND COOPERATIVE INTERACTIONS*

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The members of the glial cell line-derived neurotrophic factor (GDNF) family signal via binding to the glycosyl phosphatidylinositol-anchored membrane proteins, the GDNF family receptors α (GFRα), and activation of cRET. We performed a detailed analysis of the binding of GDNF and neurturin to their receptors and investigated the influence of cRET on the binding affinities. We show that the rate of dissociation of 125I-GDNF investigated the influence of cRET on the binding affinities. We show that the rate of dissociation of 125I-GDNF from GFRα1 is increased in the presence of 50 nm GDNF, an effect that can be explained by the occurrence of negative cooperativity. Scatchard plots of the ligand concentration binding isotherms reveal a pronounced downward curvature at low 125I-GDNF concentrations suggesting the presence of positive cooperativity. This effect is observed in the range of GDNF concentrations responsible for biological activity (1–20 nm) and may have an important role in cRET-independent signaling. A high affinity site with a KD of 11 pm for 125I-GDNF is detected only when GFRα1 is co-expressed with cRET at a DNA ratio of 1:3. These results suggest an interaction of GFRα1 and cRET in the absence of GDNF and demonstrate that the high affinity binding can be measured only when cRET is present.

Gial cell line-derived neurotrophic factor (GDNF),1 neurturin (NTN), persephin, and artemin are members of the transforming growth factor β superfamily. Their neurotrophic activity has been described in neuronal populations from the central and peripheral nervous systems (1–3). These neurotrophic factors bind to members of the family of the glycosyl phosphatidylinositol-anchored membrane protein, GDNF family receptor α (GFRα) (4). This GFRα-ligand complex, together with the tyrosine kinase receptor (cRET) forms a functional receptor that activates downstream signal transduction pathways (5–8). The binding of these neurotrophic factors to GFRα1–4 receptors and activation of cRET has been investigated by a number of groups. These investigations revealed different binding affinities of the natural ligands toward the different GFRα receptors (9–11).

GFRα1 was initially described as a high affinity receptor for GDNF (9, 10). Jing et al. (9) described two affinity sites of 125I-GDNF binding to the GFRα1 receptor with KD values of 2.3 pm for the high and 170 pm for the low affinity site. Co-expression of GFRα1 and cRET in Neuro-2a cells had little influence on the two affinity sites suggesting a small or no effect of cRET on equilibrium binding. Using a cell-free binding assay, Klein et al. (11) observed one high affinity site with a KD of 3 pm. Another single binding site (KD = 63 pm) was described by Treanor et al. (10) using CHO cells stably expressing GFRα1. The binding of NTN to GFRα1 has produced the following diverse results. Using Neuro-2a cells, binding of iodinated NTN to the GFRα1 was demonstrated (12). However, using a cell-free system, Klein et al. (11) was unable to detect specific binding of iodinated NTN to GFRα1, although NTN was displacing iodinated GDNF from GFRα1 at concentrations higher than 1 nM. Similarly, in the functional assays, some groups could demonstrate cRET activation mediated by GFRα1 and NTN in NIH cells (13, 14), whereas Buj-Bello et al. (15) presented results suggesting that in neuronal cells, NTN is unable to stimulate cRET via GFRα1. GFRα2 was described as a high affinity receptor for NTN (KD = 10 pm) and binding of 125I-GDNF to this receptor was not observed (11). In contrast, using a cell-free binding assay, Sanicola et al. (16) described the binding of 125I-GDNF to both GFRα1 and GFRα2, but binding to GFRα2 was only detectable in the presence of cRET (17). Different groups (18–22) have reported the cloning of the GFRα3 receptor. The ligand for GFRα3, artemin, was recently shown to bind to the GFRα3 receptor and to be a survival factor for different sensory and sympathetic neurons (23). The latest member of this growing family is GFRα4 (24). It was reported that persephin binds to GFRα4 and that co-expression of GFRα4 and cRET results in a receptor complex that can be activated by persephin and not by GDNF and NTN (25).

In this report we describe the first detailed characterization of 125I-GDNF and 125I-NTN binding to the GFRα1 and GFRα2 receptors. We have established conditions for the binding of GDNF and NTN to the full-length receptors expressed in Chinese hamster ovary (CHO) cells and to recombinant fusion proteins, thus permitting a detailed pharmacological characterization. This study demonstrates that the presence of cold GDNF influences the rate of dissociation of iodinated GDNF. Further, we provide evidence for positive and negative cooperative interactions and propose a binding model that includes the cooperativity and the influence of cRET on binding kinetics. Our investigations demonstrate that the presence of the high affinity site can only be detected if a sufficient amount of cRET is present.

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§ The abbreviations used are: GDNF, glial cell line-derived neurotrophic factor; NTN, neurturin; GFRα, GDNF family receptor α; CHO, Chinese hamster ovary; PCR, polymerase chain reaction; SPA, scintillation proximity assay; SPr, surface plasmon resonance.
EXPERIMENTAL PROCEDURES

Materials—125I-GDNF (2100–2400 Ci/mmol) and 125I-NTN (2100–5500 Ci/mmol) were custom labeled by Amersham Pharmacia Biotech. Recombinant human GDNF and the expression vector Signal pIG plus and for the COOH-terminal hydrophobic region involved in glycosyl phosphatidylinositol-anchoring were amplified by PCR using primers incorporating HindIII and BamHI restriction sites at the 5' and 3' ends, respectively. The resulting products were cloned in-frame in the expression vector Signal plg plus. The inserts of all constructs were confirmed by sequence analysis. The results were cloned into pCMV-hCD33 plasmid, which added not less than 3 mg at the amino terminus of the mature NTN protein. NTN expression and purification were performed as described previously (14).

Expression of GFRα1-2 and Fusion Proteins in CHO Cells—CHO cells were routinely cultured in Dulbecco’s modified Eagle’s medium/F12 medium supplemented with 10% heat-inactivated fetal calf serum. Cells were transfected with GFRα1 cloned into pcDNA, using a calcium phosphate precipitation method or other transfection techniques. HeLa cells were transfected with cRET in pIREShygro, and GFRα1-2 in Signal plg plus were performed using an optimized LipofectAMINE PLUS method. For this, a total amount of 6.5 μg of DNA was incubated with 17.5 μg of PLUS reagent in 750 μl of serum-free medium for 15 min at room temperature. LipofectAMINE was diluted 50-fold into serum-free medium culture medium; 750 μl of this mixture was added to the DNA solution. Following a 15-min incubation at room temperature, 3.5 ml of serum-free medium was added, and the mixture was brought onto the cells (in a 100-mm Petri dish). The cells were incubated for 3 h at 37°C in 5% CO2, after which a 5-ml culture medium, containing 20% heat-inactivated fetal calf serum, was added. 24 h later, the medium was changed into regular culture medium. Transfection efficiencies using these optimized conditions were typically ~50%. For permanent transfections the selection medium contained either 800 μg of G418 or 800 μg of G418 and 800 μg of hygromycin. Antibiotic-resistant clones were expanded and assayed for GFRα1 expression by binding to 125I-GDNF. A CHO clone permanently expressing GFRα1 was used for generation of a GFRα1/cRET-expressing clone. Specifics were assayed for cRET expression or specific expression using specific antibodies. For all experiments, transiently transfected cells were used 48 h following a transfection.

For purification of GFRα-Fc fusion proteins, CHO cells permanently expressing fusion proteins were grown in serum-free medium (HyQ CCM5) that was collected every 3 days. Medium was centrifuged and applied to a recombinant protein A column. Bound protein was eluted with 0.1 M sodium citrate, pH 3.0, and collected into 1 x Tris buffer, pH 8.4 (dilution ratio 1:6). Protein concentration was estimated by absorbance at 280 nm using an extinction coefficient of 1.5.

Characterization of GDNF and Neurturin Binding—Radioligand, Cell-based Binding Assays—For cell suspension binding experiments, CHO cells were collected, counted, and transferred to prewetted Multiscreen filter plates (MW 96, Millipore, 0.65 μm). Routinly, 1 × 106 – 12×106 cells were used per well. Cells were incu- bated with 0.5 ml of binding buffer (Dulbecco’s modified Eagle’s medium containing 25 mM HEPES and 2 mg/ml bovine serum albumin, pH 7.4) containing 125I-GDNF or 125I-NTN with or without cold ligands for 2 h at 4°C. Following the 2-h incubation, plates were transferred onto a Multiscreen vacuum filtration manifold, and supernatants were filtered. Cells were washed two times with washing buffer (50 mM Tris-HCl, 120 mM NaCl, pH 7.4), and filters were punched out and counted in a γ counter. In some experiments transiently transfected cells were used. Following transient transfection, binding was performed 48 h later as described above.

Binding Kinetics—The rate of association of GDNF to GFRα1 or GFRα1/cRET was determined using transiently transfected cells (GFRα1/cRET DNA transfection ratio 1:1). Whole cells were incubated at 4 °C with 50 pM 125I-GDNF in the presence of a 1000-fold excess of cold GDNF. At different time points after the addition of 125I-GDNF, samples were filtered on a Multiscreen vacuum filtration manifold. The rate of dissociation was determined at 4 °C in two different ways, by adding excess cold ligand and by a large dilution of assay medium. For each method, permanently transfected cells were incubated with 50 pM GDNF for 2 h to achieve binding equilibrium. To initiate displacement, a 1000-fold excess of cold GDNF was added at different time points, and samples were filtered on a Multiscreen vacuum filtration manifold. For the dilution method, cells preincubated for 2 h were diluted 100-fold with cold incubation medium and filtered using a single filter filtration flask and 25-μm Durapore filters, 0.65 μm at different time points after dilution.

Surface Plasmon Resonance Analyses—Surface plasmon resonance (SPR) analyses were performed at 25°C with a BIAcore 2000 biosensor. The carboxylated matrix of a F sensor chip was first activated with a 1:1 mixture of 400 mM N-ethyl-N-(dimethylaminopropyl)-carbodiimide and 100 mM N-hydroxysuccinimide. Then, recombinant GDNF or NTN was applied on the activated surface in HEPES-buffered saline (150 mM NaCl, 3.5 mM EDTA, 0.05% P-20, 10 mM HEPES, pH 7.4). Unoccupied reactive groups were deactivated with 1 M ethanolamine HCl. For kinetic experiments, soluble fusion proteins were applied at concentrations of 0.8–25 nM for GFRα1-Fc and 1.5–100 nM for GFRα2-Fc in the presence or absence of GDNF. GFRα1-Fc was applied on the activated surface in HEPES-buffered saline (150 mM NaCl, 3.5 mM EDTA, 0.05% P-20, 10 mM HEPES, pH 7.4). GFRα1-Fc and GFRα2-Fc were perfused over the immobilized ligands at a flow rate of 10 μl/min for 2 min. The dissociation was monitored for 3 min followed by regeneration with 5 mM NaOH. The BIAcore evaluation software, 3.0.1 was used to calculate the association rate (k1), dissociation rate (k2), and the apparent equilibrium dissociation constant (Kd). Data on equilibrium binding were analyzed by nonlinear
Characterization of GDNF and Neurturin Binding

FIG. 1. Association and dissociation kinetics of $^{125}$I-GDNF and $^{125}$I-NTN to membrane-anchored GFRα receptors measured using whole cells at 4 °C. Association kinetics were measured on the receptors transiently expressed in CHO cells as described under “Experimental Procedures.” A, association kinetics of $^{125}$I-GDNF (50 pM) binding to GFRα1-expressing CHO cells (○) and GFRα1/cRET-expressing CHO cells (○). B, association kinetics of $^{125}$I-NTN (50 pM) binding to GFRα2-expressing CHO cells (○) and GFRα2/cRET-expressing CHO cells (○). Data were obtained in two to three separate experiments. Dissociation experiments were set up using stably transfected CHO cells as described under “Experimental Procedures.” Dissociation was induced by 50 nm cold GDNF (●), by dilution (○) or by 50 nm cold NTN (●). C, dissociation of $^{125}$I-GDNF from GFRα1-expressing CHO cells. D, dissociation of $^{125}$I-GDNF from GFRα1/cRET-expressing CHO cells. E, dissociation of $^{125}$I-NTN from GFRα2-expressing CHO cells. F, dissociation of $^{125}$I-NTN from GFRα2/cRET-expressing CHO cells. Total and nonspecific binding was measured in duplicate at each time point.

At 4 °C. The association curves are shown in Fig. 1, A–B. The observed rate of association ($k_{o b s}$) of $^{125}$I-GDNF with GFRα1 receptors transiently expressed in CHO cells was $8.9 \times 10^{-4}$ s$^{-1}$ (Table I). A similar observed rate of association of GDNF was obtained with GFRα1/cRET receptors. The DNA ratio used for transient expression of GFRα1 and cRET was 1:1. The association rate of NTN with GFRα2 and NTN with GFRα2/cRET were in the same range (Table I). To study dissociation, intact CHO cells permanently expressing GFRα1 and GFRα2 with or without cRET were incubated with a radiolabeled ligand for 2 h at 4 °C, and dissociation was induced either by an addition of excess of cold ligand or by dilution. The dissociation curves are shown in Fig. 1, C–F. Dissociation, induced by the addition of unlabeled GDNF (50 nm) was very rapid. The derived rate of dissociation ($k_d$) of $^{125}$I-GDNF from GFRα1 receptor was $9.1 \times 10^{-3}$ s$^{-1}$ (Table I). In contrast, the rate of dissociation initiated by a 100-fold dilution was 10 times slower. The rate of dissociation obtained with the GFRα1-cRET receptor complex was in the same range. The dissociation constants obtained with $^{125}$I-NTN and GFRα2 receptors, measured by the addition of unlabeled NTN (50 nm) were much slower in comparison to $^{125}$I-GDNF dissociation from GFRα1 receptors (Table I). When the rate of dissociation was determined by dilution, it was also very slow, with a $t_{1/2}$ of more than 130 min for both GFRα2 and GFRα2/cRET receptor complexes (Table I).

**Equilibrium Binding Studies**—Equilibrium saturation isotherms of $^{125}$I-GDNF binding to cells expressing either GFRα1 alone or with cRET were determined using intact cells in suspension at 4 °C. Equilibrium saturation binding isotherms are shown in Figs. 2 and 3. When GFRα1 was expressed alone either transiently or as a permanent clone, only a low affinity site was observed (Table II), with apparent $K_d$ values of 613 and 317 pm, respectively. The binding levels ($B_{max}$ values) were 571 and 273 fmol/mg protein, respectively. A higher level of binding was observed with cells permanently expressing GFRα1 and cRET ($B_{max} = 981$ fmol/mg protein) with a $K_d$ of 356 pm (Fig. 3B and Table II). When GFRα1 was transiently co-expressed with cRET at DNA ratios GFRα1/cRET 3:1 and 1:3, the affinity was increasing with an increased amount of cRET-cDNA (Fig. 2, B–D), whereas the $B_{max}$ declined (Table II). Although no high affinity site with a $K_d$ below 20 pm was observed, Scatchard analysis of the binding in the permanently and transiently expressing cell lines showed curvilinear plots at concentrations below 10 pm. These results suggest the presence of positively cooperative interactions at low concentrations of the ligand. Only when the DNA ratio in transiently transfected cells was 1:3, was there an indication of the presence of a small number of GFRα1-cRET receptor complexes (Fig. 2D). To confirm the presence of the high affinity site with this DNA ratio, a detailed analysis of binding was performed at concentrations of $^{125}$I-GDNF below 20 pm (Fig. 2, E–F). With a GFRα1/cRET DNA ratio of 1:3, the high affinity site with a $K_d$ of 11 pm and a $B_{max}$ of 12 fmol/mg protein was revealed. In contrast, a DNA ratio of 3:1 did not result in a saturable binding at these low concentrations of iodinated GDNF (result not shown). These data clearly demonstrate the presence of the high affinity site when cRET is expressed in a sufficient amount. Only when a sufficient number of cRET receptors is present, interaction of cRET and GFRα1 results in the presentation of a small number of GFRα1-cRET receptor complex in a high affinity conformation. Control experiments with varying amounts of GFRα1 alone (1–6.5 μg) did not result in a saturable binding at low concentrations of iodinated GDNF, suggesting that changes in GFRα1 protein levels are not responsible for the presence of the high affinity site.
Radioligand binding studies were performed as described under “Experimental Procedures.” Association and dissociation equilibrium constants were derived from individual curves. Mean curves are shown in Fig. 1.

Concentration binding isotherms were best fitted to a one-site model using nonlinear regression analysis.

Table I

| Receptor | Ligand | $k_{on}$ | $t_{1/2}$ | $k_{off}$ | $t_{1/2}$ | Dilution | $K_d$ | $B_{max}$ |
|----------|--------|---------|---------|---------|---------|---------|------|---------|
| GFRα1    | GDNF   | $8.9 \times 10^{-4}$ | 779     | $9.1 \times 10^{-3}$ | 76      | $9.0 \times 10^{-4}$ | 476  |
| GFRα1/cRET | GDNF  | $11.7 \times 10^{-4}$ | 603     | $5.4 \times 10^{-3}$ | 128     | $6.4 \times 10^{-4}$ | 1091 |
| GFRα2    | NTN    | $2.5 \times 10^{-4}$  | 2722    | $1.3 \times 10^{-4}$ | 5568    | $7.3 \times 10^{-5}$ | 9561 |
| GFRα2/cRET | NTN   | $2.7 \times 10^{-4}$  | 2981    | $1.1 \times 10^{-4}$ | 6144    | $8.5 \times 10^{-5}$ | 8141 |

Fig. 3. Saturation binding isotherms and Scatchard plots of $^{125}$I-GDNF binding to GFRα1 and GFRα1/cRET receptors using permanently transfected CHO cells. Binding was performed on whole cells with a range of $^{125}$I-GDNF concentrations for 2 h at 4°C as described under “Experimental Procedures.” Nonspecific binding was determined in the presence of a 500-fold excess of cold GDNF. The data represent a typical saturation experiment (A) and derived Scatchard plots (B) with CHO cells permanently expressing GFRα1 (○) and GFRα1/cRET (○). Each data point represents the mean of three values. Concentration binding isotherms were best fitted to a one binding site model using nonlinear regression analysis.

Table II

| Receptor | DNA ratio | $K_d$ | $B_{max}$ |
|----------|-----------|------|---------|
| GFRα1/pcDNA3 | 1:1  | 613  | 571    |
| GFRα1/cRET | 3:1    | 103  | 150    |
| GFRα1/cRET | 1:3    | 95   | 72     |
| GFRα1/cRET (1–20 pM) | 1:3 | 11  | 12     |
| GFRα1 (stable expression) | 1:1 | 317  | 273    |
| GFRα1/cRET (stable expression) | 356 | 981  |

GFRα1/cRET. The binding was inhibited concentration dependently in the range of 0.1 to 100 nM by unlabeled GDNF or NTN. The IC$_{50}$ values obtained with cold GDNF and NTN are summarized in Table III. From the inhibition binding curves it can also be seen that the binding of $^{125}$I-GDNF was enhanced for up to 20–25% as compared with radioligand alone, in the presence of either cold GDNF or cold NTN in a concentration range of 1–100 pM. To investigate the influence of cRET in competition binding studies, CHO cells were transiently transfected with different DNA ratios of GFRα1 and cRET. Fig. 5 shows the effect of cRET on IC$_{50}$ values for GDNF, when co-expressed with GFRα1 at different DNA ratios. The IC$_{50}$ values obtained with cold GDNF and NTN are summarized in Table III. When GFRα1 was expressed alone or with cRET at a DNA ratio of 3:1, there was an increase of bound $^{125}$I-GDNF in the presence of low concentrations of competitive GDNF. In addition, the co-expression of GFRα1 with cRET at ratios 1:1 and 1:3 resulted in biphasic inhibition curves. The co-expression ratio of 1:1 revealed the presence of a high affinity site with an IC$_{50}$ of 10.6 ± 2.1 pM and low affinity site with an IC$_{50}$

FIG. 2. Saturation binding isotherms and Scatchard plots of $^{125}$I-GDNF binding to GFRα1 and GFRα1/cRET receptors transiently expressed CHO cells. Binding was performed on whole cells with a range of $^{125}$I-GDNF concentrations for 2 h at 4°C as described under “Experimental Procedures.” Nonspecific binding was determined in the presence of a 500-fold excess of cold GDNF. CHO cells were transiently transfected with different DNA ratios of GFRα1 and cRET: GFRα1/pcDNA3 1:1 (○), GFRα1/cRET 3:1 (○), GFRα1/cRET 1:3 (□), and GFRα1/cRET 1:3 (▲). The results represent a typical saturation experiment (A) with its derived Scatchard plots (B–D). A separate saturation experiment in the low pM range with its derived Scatchard plot is shown in E and F. Each data point represents the mean of three values. Concentration binding isotherms were best fitted to a one site binding model using nonlinear regression analysis.

**Competition Binding Studies on GFRα-expressing Cells**—Initial equilibrium binding studies using CHO cells transiently expressing GFRα1 or GFRα2 demonstrated that $^{125}$I-GDNF binds specifically to GFRα1 only and that $^{125}$I-NTN binds specifically only to GFRα2. GDNF binding to GFRα2 could not be detected even in the presence of cRET (DNA transfection ratio 1:1, data not shown). Fig. 4 shows the binding of 50 pm $^{125}$I-GDNF to CHO cells that permanently express GFRα1 or GFRα1/cRET. The binding was inhibited concentration dependently in the range of 0.1 to 100 nM by unlabeled GDNF or NTN. The IC$_{50}$ values obtained with cold GDNF and NTN are summarized in Table III. From the inhibition binding curves it can also be seen that the binding of $^{125}$I-GDNF was enhanced for up to 20–25% as compared with radioligand alone, in the presence of either cold GDNF or cold NTN in a concentration range of 1–100 pM. To investigate the influence of cRET in competition binding studies, CHO cells were transiently transfected with different DNA ratios of GFRα1 and cRET. Fig. 5 shows the effect of cRET on IC$_{50}$ values for GDNF, when co-expressed with GFRα1 at different DNA ratios. The IC$_{50}$ values obtained with cold GDNF and NTN are summarized in Table III. When GFRα1 was expressed alone or with cRET at a DNA ratio of 3:1, there was an increase of bound $^{125}$I-GDNF in the presence of low concentrations of competitive GDNF. In addition, the co-expression of GFRα1 with cRET at ratios 1:1 and 1:3 resulted in biphasic inhibition curves. The co-expression ratio of 1:1 revealed the presence of a high affinity site with an IC$_{50}$ of 10.6 ± 2.1 pM and low affinity site with an IC$_{50}$.
of 2.3 ± 1.1 nM, with 18% of binding sites displaying high affinity for GDNF. The co-expression ratio of 1:3 also revealed the presence of two affinity states for GDNF, although with more pronounced effects. An IC50 for the high affinity site was 1.1 ± 0.5 nM (4). The percentage of binding sites displaying the high affinity increased to 33%. In a control Western blot experiment, performed on fusion protein-coated SPA beads, we detected specific binding of 50 pM 125I-GDNF to both GFRα1 and GFRα2 receptors using whole cells.

**Competition Studies on GFRα1-Fc Fusion Protein-coated SPA Beads**—To further investigate the specificity of 125I-GDNF and 125I-NTN binding to GFRα1 and GFRα2 receptors, respectively, we used purified GFRα1-Fc fusion proteins coated to SPA beads. Using GFRα1-Fc-coated SPA beads, we detected specific binding of 50 pm 125I-GDNF to both GFRα1 and GFRα2 (Fig. 6). The IC50 values obtained with cold GDNF and NTN are summarized in Table IV. Specific binding of 125I-NTN was also detected to both GFRα1 and GFRα2 with an important difference (Fig. 7 and Table IV). Binding of 50 pm 125I-NTN to GFRα1 was significantly lower in comparison to GFRα2. For identical amounts of fusion proteins coated, the difference in specific binding was 2.3 ± 1.1 nM, with 18% of binding sites displaying high affinity for GDNF. The co-expression ratio of 1:3 also revealed the presence of two affinity states for GDNF, although with more pronounced effects. An IC50 for the high affinity site was 1.1 ± 0.5 nM (4). The percentage of binding sites displaying the high affinity increased to 33%. In a control Western blot experiment, performed on fusion protein-coated SPA beads, we detected specific binding of 50 pM 125I-GDNF to both GFRα1 and GFRα2 receptors using whole cells.

**Table III**

| Receptor          | DNA ratio | IC50 GDNF | IC50 NTN |
|-------------------|-----------|-----------|----------|
| GFRα1/pcDNA3      | 1:1       | 1.9 ± 0.2 nM (3) | 24       |
| GFRα1/cRET       | 3:1       | 1.9 ± 0.5 nM (4) |          |
| GFRα1/cRET       | 1:1       | 10.6 ± 2.1 pM (4) | 39       |
| GFRα1/cRET (stable expression) | 1:3       | 2.3 ± 1.1 pM (4) |          |
| GFRα1/cRET (stable expression) | 1.3       | 3.8 ± 0.7 nM (5) | 9.0 ± 2.3 (3) |

**Figure 4**

Inhibition of 125I-GDNF binding to membrane-anchored GFRα1 and GFRα1/cRET receptors using whole cells. Binding was performed on whole cells using 50 pm 125I-GDNF and CHO cells permanently expressing GFRα1 or GFRα1/cRET as described under “Experimental Procedures.” A, inhibition of 125I-GDNF binding to GFRα1-expressing CHO cells by cold GDNF (■) and NTN (□). B, inhibition of 125I-GDNF binding to GFRα1/cRET-expressing CHO cells by cold GDNF (■) and NTN (□). Each data point represents the mean of three to five independent determinations ± S.E. For each experiment the pIC50 value was derived from curve fitting using a nonlinear regression analysis.

**Figure 5**

Inhibition of 125I-GDNF binding to membrane-anchored GFRα receptors using whole cells. Binding was performed on whole cells using 50 pm 125I-GDNF and transiently expressing CHO cells as described under “Experimental Procedures.” CHO cells were transfected with different DNA ratios of GFRα1 and cRET; GFRα1 (A), GFRα1/cRET 3:1 (B), GFRα1/cRET 1:1 (C), and GFRα1/cRET 1:3 (D). Each data point represents the mean of three to four independent determinations ± S.E. For each experiment the pIC50 value was derived from curve fitting using a nonlinear regression analysis.

**Figure 6**

Inhibition of 125I-GDNF binding to GFRα1-Fc and GFRα2-Fc fusion proteins adsorbed to SPA beads. Binding was performed on fusion protein-coated SPA beads using 50 pm 125I-GDNF as described under “Experimental Procedures.” A, inhibition of 125I-GDNF binding to GFRα1-Fc by cold GDNF (■) and NTN (□). B, inhibition of 125I-GDNF binding to GFRα2-Fc by cold GDNF (■) and NTN (□). Each data point represents the mean of three to seven independent determinations ± S.E. For each experiment the pIC50 value was derived from curve fitting using a nonlinear regression analysis.

125I-NTN binding was 3-fold. The addition of cold NTN above 100 nM, increased binding of labeled NTN to both GFRα1 and GFRα2. In comparison to NTN, GDNF was only able to displace partially 125I-NTN binding to GFRα2. Already at concentrations above 10 nM, GDNF increased binding of labeled NTN to GFRα1 and GFRα2 receptors.

**Surface Plasmon Resonance Binding Studies**—Additional binding experiments were performed using a surface plasmon resonance technique. In these experiments a BIAcore F1 chip was coated with GDNF and NTN and superfused with soluble forms of GFRα1-Fc and GFRα2-Fc fusion proteins. Table V shows the summary of the derived apparent binding constants. In the SPR experiment where dissociation is initiated by dilution, the rate of dissociation of 125I-GDNF was very similar to the filtration assay with a dilution initiated dissociation.
Characterization of GDNF and Neurturin Binding

Radioligand binding studies were performed as described under “Experimental Procedures.” IC\textsubscript{50} values were derived from individual curves. The results are mean IC\textsubscript{50} values ± S.E. for three to seven (n) independent experiments. Mean inhibition curves are shown in Figs. 6 and 7.

| Receptor | Ligand      | IC\textsubscript{50} GDNF | IC\textsubscript{50} NTN |
|----------|-------------|---------------------------|-------------------------|
| GFRα1    | 125I-GDNF   | 1.8 ± 0.6 (7)             | 77.1 ± 60 (3)           |
| GFRα1    | 125I-NTN    |                           |                         |
| GFRα2    | 125I-GDNF   | 3.5 ± 1.2 (5)             | 8.0 ± 2.8 (5)           |
| GFRα2    | 125I-NTN    |                           |                         |

The dissociation rate of 125I-NTN determined by SPR was not in agreement with the rate constant obtained in a cell-based assay. It was much faster, with \( t_{1/2} = 770 \) s for GFRα2. The reason for this discrepancy may be a higher sensitivity of NTN binding kinetics to the assay temperature. The association rates obtained with GFRα1-Fc and GFRα2-Fc fusion proteins and SPR were much faster in comparison to the cell-based assay, 1.0 × 10\(^{-2}\) (s\(^{-1}\)) for GFRα1 to GDNF and 1.8 × 10\(^{-2}\) (s\(^{-1}\)) for GFRα2 to NTN. The binding specificities of GFRα1 and GFRα2 for GDNF and NTN were also different from whole cell or SPA binding assays. We have found that soluble GFRα1 binds to both GDNF and NTN with an apparent \( K_D \) of 627 nM for GDNF and 1.0 nM for NTN. Soluble GFRα2 binds only to NTN with an apparent \( K_D \) of 0.9 nM. In contrast to the SPA assay where 125I-GDNF binding to GFRα2 was detected, significant binding of GFRα2 to GDNF could not be detected with SPR.

**DISCUSSION**

The current view on the signal transduction mechanism of GDNF is based on an initial binding of GDNF to its receptor, GFRα1. After the binding of GDNF to GFRα1, this complex is able to interact with cRET and induces its activation (9–10). More recently, it has been shown that GFRα1 and GFRα2 subunits are able to modulate cRET tyrosine phosphorylation in the absence of neurotrophic factors, thus suggesting that GFRα1 and cRET can interact without the prior binding of GDNF (17). Other authors have also demonstrated that GFRα1 and cRET can interact in the absence of ligand (16, 34). Our own data indicate that GFRα1–2 receptors are able to decrease the constitutive levels of cRET phosphorylation in CHO cells (data not shown). Here we report a detailed binding characterization of GDNF and NTN to GFRα1 and GFRα2. Our results support a signal transduction model in which interactions of cRET and GFRα subunits affect the affinity for GDNF and therefore suggest complex formation prior to GDNF binding. Our data also demonstrate that the high affinity sites in the low pM range can be detected only in the presence of sufficient cRET.

The results in this study show complex binding characteristics of GDNF to GFRα1. Initial experiments measuring binding kinetics showed that the dissociation rate of GDNF from GFRα1 is accelerated 10 times in the presence of cold GDNF, which can be interpreted as an evidence for negative cooperativity by GDNF for its receptor (Fig. 1). Cold GDNF re-occupy receptor when labeled GDNF dissociates, thus an exchange of unlabeled for labeled GDNF at receptor binding sites occurs. Potential experimental artifacts such as rebinding of labeled GDNF after dilution or isotopic dilution of labeled GDNF by cold GDNF in an unstirred layer at the cell surface cannot be completely excluded (28). However, cRET co-expression with GFRα1 at a DNA ratio of 1:1 did not affect this negative cooperativity. The presence of negative cooperative interactions have already been described for insulin binding to the insulin-receptor complex and also for NGF and BDNF binding to Trk receptors (26–29). In contrast, the dissociation experiment with NTN and GFRα2 showed very slow dissociation in the presence of 50 nM cold NTN. These results demonstrate a substantial difference in binding characteristics between GDNF–GFRα1 and NTN–GFRα2 complexes. The association rates of both GDNF and NTN were similar and were not influenced by the presence of cRET, when expressed transiently at a DNA ratio of 1:1.

To determine the binding affinity of GDNF to GFRα1 and to investigate further the influence of cRET, we have used in our study a whole cell binding assay with CHO cells expressing permanently or transiently GFRα1 with or without cRET. By using transient transfection, we were able to vary the relative ratio of GFRα1 and cRET CDNAs and to compare results to permanent clones. Equilibrium saturation isotherms revealed the presence of a high affinity site in the range of 300–600 pM in CHO cells permanently expressing GFRα1 (Fig. 3). We have obtained a similar affinity using a permanent clone that ex-
presses GFRα1 and cRET. In both permanent clones, we were unable to detect the presence of a high affinity site in the lower pM range (9, 10). Moreover, Scatchard analysis of equilibrium saturation isotherms revealed the presence of downward curvilinearity at concentrations below 10 pM. Despite the variability of binding data at this low pM range, this curvilinearity was always reproducible between different experiments. One possible explanation for these results is the presence of positive cooperativity at this low pM range. Such positive cooperative interactions were already described at insulin receptors and TrkA receptors (30, 31). It is also possible that much longer times are needed to reach an equilibrium at concentrations below 20 pM. In subsequent experiments we have varied the transient expression level of cRET by using different DNA ratios of GFRα1 and GFRα1/cRET of 3:1 and 1:3. Using either GFRα1 alone or with cRET at a DNA ratio of 3:1, equilibrium saturation isotherms have also revealed the presence of a high affinity site in the range of 100 pM with a downward curvilinearity in the Scatchard plot (Fig. 2, A, C). Surprisingly, using a DNA ratio of 1:3, an increased affinity was obtained, and the presence of a small percentage of high affinity sites was detected (Fig. 2D). More detailed equilibrium saturation isotherms in the low pM range clearly demonstrated the presence of this high affinity site (Fig. 2, E and F). These results suggest that the high affinity site is present only if a sufficient amount of cRET receptors is present on the cell surface. In the same experimental conditions, no positive cooperativity was observed. The mechanism for this observation remains unknown. If GFRα1 is expressed alone or cRET expression is relatively low, positive cooperative interactions can be seen.

These results are not in agreement with earlier studies where low and high affinity sites were reported in cells expressing either GFRα1 or GFRα1/cRET (9, 10). Using 293T cells expressing GFRα1 and a whole cell-based binding assay, Jing et al. (9) reported a high affinity site with a Kd value of 2.3 pM and low affinity site with a Kd value of 170 pM. These affinities were not influenced by the presence of cRET; Kd values of 1.5 and 332 pM were found in Neuro 2A cells expressing both proteins. The authors reported the presence of low levels of cRET mRNA in 293T cells, and we have also found endogenous cRET mRNA in HEK 293 cells (data not shown). These low levels of cRET mRNA may be sufficient to produce enough cRET protein to influence the affinity of iodinated GDNF. It has also been shown that CHO cells under certain experimental conditions can express low levels of neurturin (2). With the experimental conditions used in this study, such as extensive washing and a 2-h incubation on ice, it is very unlikely that endogenous NTN could influence the affinity of iodinated GDNF.

To further investigate the influence of cRET, competition binding studies were performed with CHO cells transiently or permanently expressing GFRα1 and cRET. Both GDNF and NTN were able to displace 125I-GDNF binding to CHO cells permanently expressing either GFRα1 or GFRα1/cRET, with low affinity sites (Fig. 4). When GFRα1 was transiently expressed alone or with cRET at a DNA ratio of 3:1, GDNF also displaced 125I-GDNF binding with a single affinity site of lower affinity than in permanent clones (Fig. 5 and Table III). Only with an increased amount of cRET cDNA, either as a DNA ratio of 1:1 or 1:3, GDNF displacement of 125I-GDNF binding fitted best a two site affinity model. At these conditions a high affinity binding site with a Kd of 2–10 pM was observed (Fig. 5, C and D). These results indicate again that the presence of a high affinity site is dependent on the expression level of cRET. In these competition experiments, an initial increase of specific binding from 100 to 125% at a low pM range of cold ligand supports the presence of positive cooperative interactions between GFRα1 receptors. With an increased expression of cRET, positive cooperativity was abolished, and cold GDNF displaced iodinated GDNF with two affinities.

Based on our data, we tentatively propose a reaction scheme to be verified by further experiments. The reaction scheme is presented in Fig. 8. Our binding scheme is based on interactions between GDNF, GFRα1, and cRET. It allows for interactions of GFRα1 and cRET in the absence of GDNF and gives the possibility to explain cooperative interactions (Fig. 8) (32). In the absence of cRET and the presence of low GDNF concentrations, ligand-induced conformational change results in positive cooperativity and has favorable effects on the binding of the subsequent ligand(s) (k2 > k1). When cRET is present in sufficient amount, its pre-association with GFRα1 results in the detection of low (RZ) and high (R*Z) affinity states of receptor complex. In the presence or absence of cRET, high concentrations of GDNF result in a conformational change of the receptor-ligand complex that has unfavorable effects on the binding of subsequent ligand(s), negative cooperativity (k3 >> k4; k’3 >> k’4).

![Proposed reaction scheme for GDNF binding to GFRα1 receptors.](https://example.com/scheme.png)
iodinated NTN to GFRα2-Fc can only be competed by cold NTN. Cold GDNF competes only with a small percentage of $^{125}$I-NTN binding. Similar observations have been reported and suggest a difference in neurotrophic factor binding pockets at GFRα1 and GFRα2 receptors (12). In addition, cold NTN and GDNF used at higher concentrations increased the binding of iodinated NTN to both GFRα1-Fc and GFRα2-Fc receptors. A similar effect could not be seen with iodinated GDNF.

In summary, we have presented the first detailed binding characterization of GDNF and NTN to GFRα1 and GFRα2 receptors. The results presented here demonstrate the complexity of GDNF interaction with its receptor underlined by the presence of positive and negative cooperative interactions. The physiological role of positive cooperativity may be to ensure full occupancy of GDNF receptors at low GDNF concentrations. Positive cooperativity occurs in the range of GDNF concentrations responsible for biological activity (1) and may have an important role in cRET-independent signaling (33). On the other hand, the role of negative cooperativity may be to attenuate the cellular response at higher concentrations of GDNF. A similar binding mechanism has been proposed for the insulin receptor (26). Finally, our model of GDNF binding to GFRα1 and GFRα2 receptors expressed in CHO cells proposes the presence of a high affinity receptor complex in the absence of GDNF is necessary for receptor complex conformation with two affinity sites.

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Binding of GDNF and Neurturin to Human GDNF Family Receptor α 1 and 2: INFLUENCE OF cRET AND COOPERATIVE INTERACTIONS

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