The first cysteine-rich domain of the receptor GFR α 1 stabilizes the binding of GDNF

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The GDNF (glial cell line-derived neurotrophic factor)-binding receptor GFR α 1 (GDNF family receptor α 1) is attached to the membrane by a GPI (glycosylphosphatidylinositol) anchor and consists of three cysteine-rich domains. The region corresponding to the second and third domains has been shown previously to participate in ligand binding, and to interact with the transmembrane tyrosine kinase receptor RET. No function has so far been found for the N-terminal, first domain (D1). Here we show that the GPI-anchored full-length receptor binds ¹²⁵I-GDNF two times more tightly than does a GPI-anchored truncated receptor lacking D1. Scintillation proximity assays with purified receptor proteins also show that the GDNF-binding capacity of the soluble full-length GFR α 1 is two times higher than the GDNF-binding capacity of the soluble D1-truncated GFR α 1. As RET stabilizes the binding of GDNF equally well to the full-length and truncated receptors, D1 seems not to be involved in the interaction between GFR α 1 and RET. Moreover, soluble full-length GFR α 1 mediates

GDNF-promoted neurite outgrowth in PC6-3 cells more efficiently than the soluble truncated GFR α 1 protein. At low concentrations, the soluble full-length receptor mediates the phosphorylation of RET more efficiently than the soluble truncated receptor. However, when the receptors are overexpressed on the cell surface as GPI-anchored proteins, or added to the growth medium at high concentrations as soluble proteins, full-length and truncated GFR α 1 are indistinguishable in GDNF-dependent RET-phosphorylation assays. High levels of the receptors can thus mask a slightly impaired function in the phosphorylation assay. Based on assays with both GPI-anchored and soluble receptors, we therefore conclude that D1 contributes to the optimal function of GFR α 1 by stabilizing the interaction between GFR α 1 and GDNF.

Key words: glial cell line-derived neurotrophic factor family receptor α 1 (GFR α 1), glial cell line-derived neurotrophic factor (GDNF), interaction, RET, structure–function.

INTRODUCTION

GDNF (glial cell line-derived neurotrophic factor) binds to the co-receptor GFR α 1 (GDNF family receptor α 1) which, upon binding of the ligand, mediates the phosphorylation of the transmembrane tyrosine kinase receptor RET. GDNF has a broad range of functions as a survival factor and a regulator for central and peripheral neurons, and as a morphogenetic factor in the kidney and spermatogonia development. It is therefore clear that the GFR α 1–RET receptor complex has potential for therapeutic applications. Indeed, in animal models, exogenously administered GDNF can support axonal regeneration after peripheral nerve injury [1], reduce ischaemic brain trauma [2,3], and prevent the degeneration of midbrain dopaminergic neurons [4]. Recently, promising results have been obtained by delivering GDNF protein directly into the putamen of patients with Parkinson's disease in a phase 1 clinical trial [5]. (For a review on the GDNF family, its signalling, biological functions and therapeutic value, see [6].)

Drug development relies on the detailed characterization of proteins involved in a receptor complex. GDNF belongs to the GDNF family of ligands, which consists of the close homologues GDNF, neurturin, artemin and persephin. These four ligands bind to the specific co-receptors GFR α 1–GFR α 4, but signal through the common RET receptor [6]. The GFR α s are GPI (glycosylphosphatidylinositol)-anchored proteins that have all,

except for mammalian GFR α 4, been postulated to consist of three similar cysteine-rich domains, D1, D2 and D3 [7–9]. In GFR α 1, the core region consisting of D2 and D3 has been shown to interact both with GDNF and RET [10,11]. However, no function has been assigned to the N-terminal D1 domain, which is missing completely from mammalian GFR α 4. GFR α 4 is the only GFR α that has been described to show differences with respect to its capacity to mediate the ligand-dependent phosphorylation of RET [12,13], to induce neurite outgrowth [14,15], and to recruit RET to the lipid rafts [16]. It is always difficult to compare the activity of different GFR α s because many parameters, such as the source of the ligands and the expression levels of the receptor, influence the results. It is, however, tempting to speculate that D1 could be important for the optimal function of the GFR α s, and that the lack of D1 may somehow reduce the activity of the GFR α .

Rat GFR α 1 consists of 468 amino acid residues, including the ER (endoplasmic reticulum) signal sequence. Previous studies with numerous truncated and chimaeric GFR α 1 constructs showed that D1 of both GFR α 1 and GFR α 2 is dispensable and does not affect the ligand-binding specificity [10]. In that particular study, the authors assayed the capacity of GFR α 1 and GFR α 2 to mediate the phosphorylation of RET, as well as to become chemically cross-linked to GDNF and neurturin. Recently, the first crystal structure analysis of part of a GFR α showed that D3 (amino acid residues 239–346) of GFR α 1 represents a novel

Abbreviations used: D1–D3, first, second and third domains respectively of GFR α 1; DMEM, Dulbecco's modified Eagle's medium; ER, endoplasmic reticulum; GDNF, glial cell line-derived neurotrophic factor; GFR α 1, glial cell line-derived neurotrophic factor family receptor α 1; GFR α 1¹²², truncated GPI-anchored GFR α 1 variant lacking residues 23–121; GFR α 1^{WT}, GPI-anchored full-length wild-type GFR α 1; GFR α 1²⁰, GFR α 1¹¹⁴, GFR α 1¹⁴⁵, soluble GFR α 1 variants comprising residues 20–425, 114–425 and 145–425 respectively of GFR α 1; GPI, glycosylphosphatidylinositol; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; N-CAM, neural cell adhesion molecule; NGF, nerve growth factor; RET^{ED}, RET extracellular domain; SPA, scintillation proximity assay.

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protein fold [11]. Based on sequence identity, D2 (residues 150–238) was modelled and used to build a model of the two-domain receptor (D2–D3). Site-directed mutagenesis and functional assays showed that the major GDNF-binding surface of this two-domain receptor is located in the cleft between D2 and D3 of GFR α 1 [11]. D1 is similar to the other domains, but it lacks one pair of cysteine residues, and is linked to D2 by a potential hinge consisting of about 30 amino acid residues [7,11]. In order to characterize the functional role of D1 in more detail, we have constructed different deletion variants of both GPI-anchored and soluble GFR α 1 and used them for direct binding assays, neurite outgrowth assays and RET-phosphorylation assays. Our results show that D1 is involved in stabilizing the binding of GDNF, thereby optimizing the function of GFR α 1.

EXPERIMENTAL

GPI-anchored GFR α 1 constructs

Throughout this paper, the numbering of the amino acid residues of rat GFR α 1 refers to the protein with GenBank accession number Q62997. Our full-length GFR α 1 construct corresponds to accession number AJ002072, which differs from Q62997 by a deletion of five amino acids (residues 140–144). According to the current structural model of GFR α 1, D2 is postulated to span amino acids 150–238 [11]. To generate the GPI-anchored, truncated GFR α 1 (designated GFR α 1¹²²), amino acid residues 23–121 were deleted. Both the full-length, wild-type (GFR α 1^{WT}) and truncated construct were tagged with a FLAG epitope (ASDYKDDDD-KAS), which was inserted immediately after the 22-amino-acid ER signal sequence. Both constructs were cloned into pcDNA3 and sequenced. Antibodies to FLAG (M2; Sigma; 1:1500) were used to verify the correct molecular masses of the constructs. The presence of N-linked glycans on both constructs was analysed by expressing the proteins in COS7 cells overnight in the presence or absence of tunicamycin (10 μ g/ml). Total lysates of the cells were prepared and analysed by Western blot analysis.

Expression and purification of soluble GFR α 1 variants and RET^{ED}

The soluble proteins were expressed using the Bac-to-Bac[®] baculovirus expression system (Gibco). The cDNA regions of full-length rat GFR α 1 (GenBank[™] AJ002072) and of truncated variants coding for amino acids 20–425 (GFR α 1²⁰), 114–425 (GFR α 1¹¹⁴) or 145–425 (GFR α 1¹⁴⁵) were amplified by PCR. GFR α 1²⁰ lacks only the ER signal sequence (see above), and therefore corresponds to full-length GFR α 1. GFR α 1¹¹⁴ lacks both the ER signal sequence and D1 (see above). The most truncated construct, GFR α 1¹⁴⁵, is devoid of the ER signal sequence, D1 and the potential hinge between D1 and D2. This hinge region has been predicted to consist of about 30 amino acids [7,11]. Therefore GFR α 1¹¹⁴ and GFR α 1¹⁴⁵ both lack D1, but differ from each other in the presence or absence of the hinge region. The primers directed to the GFR α 1 sequence were designed to exclude the GPI anchor signal sequence (by a stop codon), to include an N-terminal His₆ tag, and to incorporate both 5'-NotI and 3'-HindIII restriction sites. The soluble rat GFR α 1 variants were cloned into the baculovirus vector pK503.9 [17], a pFastBac derivative (Gibco), which added a FLAG tag and an insect cell secretion signal sequence at the N-terminus. The GFR α 1 variants were expressed in Sf9 insect cells after baculovirus infection at high multiplicity. Sf9 cells were grown in serum-free SF900II medium (Invitrogen) supplemented with 50 μ g/ml gentamycin (Sigma) at 27°C. At 3 days post-infection, GFR α 1 variants were purified from culture supernatants by Ni-chromatography (Ni²⁺-charged

Chelating Sepharose; Amersham Biosciences). The supernatant was adjusted to pH 7.5 with PBS and Ni²⁺-resin was added. After a 2 h incubation at 4°C, the resin was washed with PBS containing 5 mM imidazole. After the imidazole elution, proteins were concentrated using Centricon (Millipore) spin concentrators and the buffer was changed to PBS (pH 7.4). Protein concentrations were estimated by SDS/PAGE analysis with Coomassie Blue staining, in comparison with known concentrations of ovalbumin and ribonuclease A (Amersham Biosciences).

The cDNA region encoding RET^{ED} (extracellular domain of human RET; GenBank X12949) was amplified by PCR. The primers were designed to encode amino acid residues 28–636 with a C-terminal His₆ tag and a stop codon at the 3'-end. The resulting product was cloned into the baculovirus vector pK503.9 [17], which added a FLAG tag and an insect cell secretion signal sequence at the N-terminus. RET^{ED} protein was expressed as above and used unpurified. The amounts of RET^{ED} were estimated by Western blotting against known concentrations of purified GFR α 1 D3, and its biological activity was verified as described previously [11].

Characterization of purified soluble receptors

To characterize the purified receptors, they were subjected to reverse-phase chromatography on a 1 mm $\times$ 150 mm Vydac C8 column (LC-Packings; 8.5 μ m, 300 Å) equilibrated with 0.1% trifluoroacetic acid. Elution was performed with a linear gradient of acetonitrile (0–100% in 60 min) in 0.1% trifluoroacetic acid at a flow rate of 50 μ l/min and monitoring at 214 nm. The receptor-containing fractions were collected and the receptors subjected to N-terminal sequence analysis using a Procise 494A sequencer (Perkin-Elmer, Applied Biosystems Division). MALDI-TOF (matrix-assisted laser desorption-ionization time-of-flight) MS was performed in the positive-ion mode with an Ultraflex TOF/TOF mass spectrometer (Bruker-Daltonik), using a 337 nm nitrogen laser and sinapinic acid as the matrix. Electrospray ionization MS was performed in the positive-ion mode using a Q-TOF hybrid mass spectrometer (Micromass).

To determine whether the purified receptors were monomeric or dimeric, the samples were analysed by gel filtration chromatography. The protein samples were run at a flow rate of 0.5 ml/min on a Superdex 200 HR 10/30 column (Amersham Biosciences). The column was equilibrated at 8°C in 50 mM sodium phosphate, pH 7.5, 500 mM NaCl. All samples (100–300 ng/ μ l) were prepared in the elution buffer and had a volume of approx. 200 μ l. Ribonuclease A (14 kDa), chymotrypsinogen (25 kDa), ovalbumin (43 kDa) and BSA (66 kDa) were used to calibrate the column. The eluant was monitored by a UV detector at a wavelength of 280 nm. For soluble GFR α 1 variants, fractions (500 μ l) were collected and assayed for GDNF-binding activity by a SPA (scintillation proximity assay; see below).

GDNF binding assays

Rat GDNF (Cephalon, Inc.) was iodinated with Na¹²⁵I using the lactoperoxidase method [18]. The cell-based binding assay was performed on transiently transfected COS7 cells as described previously [13]. At 24 h after transfection, the cell surface was blocked with 0.5% (w/v) dried milk and 0.2% (w/v) BSA in DMEM (Dulbecco's modified Eagle's medium)/15 mM Hepes (pH 7.4). The ¹²⁵I-labelled GDNF (50 pM) and unlabelled GDNF (0–5000 pM) were added in the same buffer. Cells were incubated on ice for 2 h, washed three times with the same buffer without milk and BSA, and lysed with 1 M NaOH. The lysates were counted for radioactivity using a 1480 WIZARD 3" scintillation counter (Wallac). For the binding assays with both GFR α 1

and RET, COS7 cells were co-transfected with the corresponding cDNAs at a ratio of 1:1. The background of each point in the assay was determined as the amount of ^{125}I -GDNF bound to RET-transfected cells. Each point of the curve represents the mean of four parallel samples.

To determine IC_{50} values, we used the non-linear regression analysis program Prism 3.02 (GraphPad Prism Software, San Diego, CA, U.S.A.). The binding data were fitted according to the one-site binding model, with the background determined at each measurement point. As the concentration of ^{125}I -GDNF used was much lower than the IC_{50} values obtained, the IC_{50} values correspond approximately to K_d (dissociation constant) values.

SPA

Binding studies were performed in 96-well plates (HB Isoplate; Wallac) in volumes of 200 μl . SPA PVT beads precoated with anti-mouse antibodies (Amersham Biosciences) were first mixed with 10 ng of anti-FLAG (M1) antibodies and 10 ng of GFR α 1²⁰ or GFR α 1¹⁴⁵ in Tris-buffered saline (pH 7.4) supplemented with 1 mM CaCl_2 and 1.0 mg/ml BSA. When needed, an excess of RET was added in 20 μl of RET^{ED}-medium. The beads were then incubated with 50 pM ^{125}I -GDNF (see above) in the presence of unlabelled GDNF (Amgen) at concentrations up to 4 nM. Background levels were defined in the presence of RET^{ED} without GFR α 1. After a 3 h incubation at room temperature, plates were counted for radioactivity in a MicroBeta Trilux scintillation counter (Wallac). SPAs were repeated four or six times with three or four parallel measurements. The IC_{50} values were calculated as above.

Neurite outgrowth assays

For the neurite outgrowth assays with soluble GFR α 1, PC6-3 cells [19] were transferred to a medium consisting of RPMI, 1% (v/v) horse serum and penicillin/streptomycin (Gibco) on plates coated with collagen (Becton Dickinson Labware). Soluble GFR α 1²⁰, GFR α 1¹¹⁴ or GFR α 1¹⁴⁵ (1000 ng/ml) plus GDNF (100 ng/ml; PeproTech) or NGF (nerve growth factor; 50 ng/ml; Promega) were added. The PC6-3 cell line is a subclone of the parental PC12 cell line, which was used previously for the same type of neurite outgrowth assay with GDNF and soluble GFR α 1 [20,21]. The neurites were counted after 3–4 days, and only those neurites longer than the diameter of the cell soma were recorded. Around 200–300 cells were counted in each sample, and the experiment was repeated three times with different batches of purified GFR α proteins. Comparisons were performed with Student's *t* test assuming unequal variances. Results are shown as means $\pm$ S.E.M., and *P* < 0.05 was regarded as statistically significant.

Phosphorylation assays

For phosphorylation assays with soluble GFR α 1²⁰, GFR α 1¹¹⁴ or GFR α 1¹⁴⁵, MG87-RET cells [22] were starved for 4 h in serum-free DMEM at 37°C, and subsequently stimulated with 100 ng/ml GDNF and 0–1000 ng/ml of the soluble GFR α 1 proteins for 60 min at 37°C. The MG87-RET cell line is stably transfected with RET, but does not express GFR α 1. After lysis, antibodies to RET (C-20; Santa Cruz Biotechnology Inc.) were added to the post-nuclear supernatant. The immunocomplexes were collected with Protein G–Sepharose (Amersham Biosciences) and analysed by Western blotting with antibodies to phosphotyrosine (4G10; Upstate Biotechnology; 1:1500). Equal loading was verified by restaining of the filters with antibodies to RET.

For phosphorylation assays with the GPI-anchored forms of GFR α 1, MG87-RET cells were transiently transfected with LipofectamineTM 2000 (Invitrogen). On the following day the cells were starved for 4 h in serum-free DMEM at 37°C, and were stimulated subsequently with 100 ng/ml GDNF for 10 min at 37°C. After lysis, the samples were treated as above. Equal expression levels of the transiently transfected GPI-anchored receptors were verified by taking samples before immunoprecipitating with antibodies to RET.

To visualize phosphorylation of endogenously expressed RET in PC6-3 cells, the cells were stimulated with 100 ng/ml GDNF and 1000 ng/ml full-length soluble GFR α 1 for 60 min at 37°C. After lysis, the samples were treated as above, except that antibodies to both isoforms of RET (C-19 and C-20; Santa Cruz Biotechnology Inc.) were used in order to maximize the amount of immunoprecipitated RET. The two isoforms of RET differ from each other only by 42 amino acid residues, and both are synthesized as intracellular precursors (~150 kDa) which mature into extracellular receptors (~170 kDa). Both antibodies were used to verify the identity the phosphorylated proteins, and to confirm equal loading of the samples.

RESULTS

Characterization of soluble and GPI-anchored receptors

To study the interactions of the soluble GFR α 1 variants, sequences encoding amino acids 20–425 (GFR α 1²⁰), 114–425 (GFR α 1¹¹⁴) and 145–425 (GFR α 1¹⁴⁵) of the rat GFR α 1 polypeptide were cloned into an insect cell expression vector for production of N-terminal FLAG and His₆ fusion proteins. Following baculovirus infection, the recombinant proteins were isolated from the harvested culture medium by Ni²⁺–Sepharose affinity purification. The purification was confirmed by anti-FLAG Western blotting, and the purified proteins were quantified on Coomassie Blue-stained SDS/PAGE against known concentrations of commercial molecular mass markers. The calculated molecular masses for the expressed GFR α 1²⁰, GFR α 1¹¹⁴ and GFR α 1¹⁴⁵ variants are 46 809 Da, 36 109 kDa and 33 147 Da respectively, but SDS/PAGE analysis showed that GFR α 1²⁰, GFR α 1¹¹⁴ and GFR α 1¹⁴⁵ ran with apparent molecular masses of approx. 54–56 kDa, 42–44 kDa and 38–40 kDa respectively (results not shown).

To characterize further the expressed receptors GFR α 1²⁰ and GFR α 1¹⁴⁵, they were subjected to reverse-phase chromatography followed by N-terminal sequence analysis and MS analysis. Each of the purified receptors eluted in reverse-phase chromatography as a single peak with an intensity corresponding to the expected amount of protein (results not shown). Both receptors collected from reverse-phase chromatography were subjected to N-terminal sequence analysis, and both gave a pure N-terminal sequence of DYKDDDDKRP..., corresponding to the suggested N-terminus of the expressed proteins. MALDI-TOF MS analysis of GFR α 1²⁰ and GFR α 1¹⁴⁵ gave molecular masses of 52 122 Da and 37 073 Da respectively, indicating that both receptors are post-translationally modified. The full-length receptor harbours a bigger modification (5314 Da) than the truncated receptor (3926 Da), indicating that D1 of GFR α 1 also harbours a modification. In the case of GFR α 1²⁰, the size of the modification could equal a glycan present on each of the three potential N-glycosylation sites, whereas the modification of GFR α 1¹⁴⁵ could represent two N-linked glycans (results not shown). In electrospray MS, the full-length receptor was ionized, but no masses could be deconvoluted from the primary mass spectrum, indicating considerable heterogeneity in the molecular mass of the protein, possibly due to heterogeneous glycan structures. For

Figure 1 Gel filtration analysis of the soluble GFR α 1 variants GFR α 1¹²⁰ (■) and GFR α 1¹⁴⁵ (▲)

Molecular masses were estimated from a calibration curve of the molecular mass standards ribonuclease A (14 kDa), chymotrypsinogen (25 kDa), ovalbumin (43 kDa) and BSA (66 kDa) (indicated by ◇). Both receptor variants eluted between the 66 kDa and the 25 kDa markers.

the truncated receptor GFR α 1¹⁴⁵ a set of heterogeneous masses around 37 kDa could be deconvoluted. The mass heterogeneity was due mainly to multiple mass differences of 203 Da and 162 Da, which are similar to the masses of *N*-acetylglucosamine and hexose respectively. Thus the heterogeneous masses obtained most probably reflect heterogeneity of the glycan part of the glycoprotein. These results are fully consistent with the earlier identification [23] of one putative *N*-glycosylation site in D1 (Asn-59) and two in the hinge connecting D3 to the cell membrane (Asn-347, Asn-406).

To determine whether the soluble receptors are monomeric or dimeric, the purified variants GFR α 1¹²⁰ and GFR α 1¹⁴⁵ were analysed by gel filtration chromatography. All fractions were analysed by Western blot (results not shown), and their GDNF-binding capacity was analysed by SPA (Figure 1). Both receptors were eluted in the fractions between the molecular mass markers BSA (66 kDa) and chymotrypsinogen (25 kDa). We therefore conclude that purified GFR α 1¹²⁰ and GFR α 1¹⁴⁵ are monomeric.

The molecular masses of the two GPI-anchored GFR α 1 constructs were determined by transiently expressing them in COS7 cells in the presence or absence of tunicamycin, an inhibitor of *N*-linked glycosylation. Sequence analyses of these constructs predicted the expression of FLAG-tagged proteins with molecular masses of approx. 53 kDa for GFR α 1^{WT} and 42 kDa for GFR α 1¹²². Our Western blot analysis with anti-FLAG antibodies showed the expression of two tagged proteins of the predicted sizes. When the receptors were expressed in the presence of tunicamycin, the molecular mass of GFR α 1¹²² was decreased by approx. 4 kDa, whereas that of GFR α 1^{WT} was decreased by approx. 6 kDa, indicating that both receptors show *N*-linked glycosylation (Figure 2). Both GPI-anchored GFR α 3 [24] and GPI-anchored GFR α 4 [16] have been shown previously to harbour *N*-linked glycans.

Cell-based binding assays

A cell-based binding assay was used to characterize whether ¹²⁵I-labelled GDNF binds differently to full-length and truncated GFR α 1. The GPI-anchored forms GFR α 1^{WT} or GFR α 1¹²² were transiently expressed on the surface of COS7 cells and the cells were used for homologous competition binding assays. Using this assay, we determined an IC₅₀ of 1.12 ± 0.14 nM (mean ± S.E.M.;

Figure 2 Expression of the two GPI-anchored GFR α 1 variants in COS7 cells in the presence or absence of tunicamycin (10 µg/ml)

$n = 4$) for the interaction of GDNF with transiently expressed GFR α 1^{WT}, and an IC₅₀ of 2.06 ± 0.40 nM for its interaction with transiently expressed GFR α 1¹²² (Figure 3A, Table 1). GFR α 1^{WT} thus binds GDNF two times more tightly than does GFR α 1¹²².

It has been reported previously that RET stabilizes the binding between GDNF and GFR α 1 [11,25]. To elucidate whether D1 contributes to the interaction with RET, we co-transfected RET with the two forms of GPI-anchored GFR α 1 (GFR α 1^{WT} and GFR α 1¹²²) and then repeated the GDNF-binding assay. The IC₅₀ for the interaction of GDNF with GFR α 1^{WT} was decreased from 1.12 ± 0.14 nM in the absence of RET to 0.49 ± 0.02 nM in the presence of RET. The IC₅₀ for the interaction of GDNF with GFR α 1¹²² was correspondingly decreased from 2.06 ± 0.40 nM in the absence of RET to 0.77 ± 0.07 nM in its presence (Figure 3B, Table 1). The presence of RET therefore stabilizes the binding of GDNF to GFR α 1^{WT} and GFR α 1¹²² by a factor of 2.3 and 2.7 respectively. We therefore conclude that D1 of GFR α 1 is important for binding of GDNF, but not for the interaction with RET.

Cell-free binding assays with purified proteins

The results from the cell-based binding assays were verified using SPAs with purified soluble receptor proteins. Each sample was assayed four to six times. The results obtained fully supported those obtained with the GPI-anchored receptors (Figures 3C and 3D, Table 2). Most importantly, this assay, which is based on purified proteins in a test tube, showed that D1 of GFR α 1 is able to stabilize the binding between GDNF and GFR α 1 in the absence of other components, such as RET, heparan sulphates [26] or N-CAM (neural cell adhesion molecule) [27]. In addition, the SPA (similarly to the cell-based binding assay) showed that RET^{ED} stabilizes the interaction between GDNF and GFR α 1 independently of D1.

Neurite outgrowth assays

The neurite outgrowth assay was performed in PC6-3 cells, as these cells form fewer aggregates than the parental PC12 cells and therefore are easier to manipulate. NGF (50 ng/ml) was used as a positive control for the experimental set-up. The neurites were counted 3–4 days after the addition of 100 ng/ml (3.3 nM) GDNF and 1000 ng/ml soluble GFR α 1²⁰, GFR α 1¹¹⁴ or GFR α 1¹⁴⁵, which corresponds to a molarity of 21 nM for GFR α 1²⁰, 27 nM for GFR α 1¹¹⁴ and 30 nM for GFR α 1¹⁴⁵. GFR α 1²⁰ was thus used at a slightly lower molar concentration than GFR α 1¹¹⁴ and GFR α 1¹⁴⁵. The assay was repeated three times with three different batches of purified soluble receptors. Only neurites longer than the cell soma were counted, and 200–300 cells were counted in each sample. Figure 4(A) shows that full-length GFR α 1²⁰ was more than 2-fold more active than the two truncated receptors GFR α 1¹¹⁴ and GFR α 1¹⁴⁵ that lack D1. GFR α 1¹⁴⁵ seemed to be more effective

Figure 3 GDNF binding assays

(A) Displacement of ^{125}I -GDNF by unlabelled GDNF from COS7 cells transiently transfected with the GPI-anchored variants GFR α 1 122 (■) or GFR α 1 $^{\text{WT}}$ (▲). (B) As in (A), but after transient co-transfection with RET. (C) Displacement of ^{125}I -GDNF by unlabelled GDNF from GFR α 1 145 (●) or GFR α 1 120 (▲)-coated SPA beads. (D) As in (C), but in the presence of RET $^{\text{ED}}$.

Table 1 Cell-based binding assays with ^{125}I -GDNF and transiently expressed membrane-bound GFR α 1 and RET proteins

Values represent means $\pm$ S.E.M. ($n = 4$).

Transiently expressed construct	IC ₅₀ (nM)
GFR α 1 $^{\text{WT}}$	1.12 $\pm$ 0.14
GFR α 1 122	2.06 $\pm$ 0.40
GFR α 1 $^{\text{WT}}$ + RET	0.49 $\pm$ 0.02
GFR α 1 122 + RET	0.77 $\pm$ 0.07

Table 2 SPAs with ^{125}I -GDNF and purified soluble GFR α 1 and RET proteins

Values represent means $\pm$ S.E.M. ($n = 4-6$).

Protein	IC ₅₀ (nM)
GFR α 1 120	0.75 $\pm$ 0.15
GFR α 1 145	1.59 $\pm$ 0.18
GFR α 1 120 + RET $^{\text{ED}}$	0.17 $\pm$ 0.04
GFR α 1 145 + RET $^{\text{ED}}$	0.43 $\pm$ 0.10

in mediating the neuritogenic activities of GDNF than GFR α 1 114 . The reason for this difference might be that the hinge region, which is normally located between D1 and D2 of GFR α 1, is N-terminally exposed in GFR α 1 114 , thereby possibly disturbing the function of the receptor. To ensure that this neurite outgrowth correlated with the phosphorylation of endogenous RET in the PC6-3 cells, we immunoprecipitated RET and monitored its phosphorylation with antibodies to phosphotyrosine. The level of

endogenously expressed RET is very low in PC6-3 cells, but the addition of GDNF and soluble GFR α 1 induced phosphorylation of the RET protein (Figure 4B).

Phosphorylation assays

RET is a glycosylated transmembrane protein that is expressed in two forms in MG87-RET cells. The 150 kDa precursor is located in the ER, while only the mature, 170 kDa form is located on the cell surface [28]. Therefore only the 170 kDa form of RET becomes phosphorylated upon extracellular stimulation with ligands. The phosphorylation assay with the transiently expressed GPI-anchored variants GFR α 1 $^{\text{WT}}$ and GFR α 1 122 showed that they mediated the phosphorylation of the 170 kDa extracellular form of RET equally well (Figure 5A). The control showed that GDNF alone does not induce any background phosphorylation in MG87-RET cells (Figure 5C); this has also been documented previously [22]. As the difference between the full-length and truncated receptors was not distinct in the phosphorylation assay, but was clear in both the binding assay and the neurite outgrowth assay, we reasoned that the phosphorylation assay might be hampered by the high expression levels of the transiently expressed GPI-anchored GFR α 1. In other words, we thought that high transient expression levels of the GFR α 1 variants could mask a slightly impaired function, and that small differences between the variants might become detectable only when the GFR α 1 number becomes limiting. To test this hypothesis, we performed phosphorylation assays with decreasing concentrations of the soluble GFR α 1 variants. At low concentrations, the full-length receptor GFR α 1 120 mediated RET phosphorylation more efficiently than the two truncated receptors GFR α 1 114 and GFR α 1 145 . GFR α 1 120 mediated maximal phosphorylation of RET

Figure 4 Neurite outgrowth and RET-phosphorylation assays in PC6-3 cells

(A) Neurite outgrowth. The soluble receptors GFR α 1²⁰, GFR α 1¹¹⁴ or GFR α 1¹⁴⁵ (1000 ng/ml) were added with or without GDNF (100 ng/ml), and the neurites (defined as being longer than the cell soma) were counted after 3–4 days. The experiment was repeated three times with parallel samples, and 200–300 cells were counted per sample. Values are means $\pm$ S.E.M.; ** P < 0.01 (Student's t test). NGF (50 ng/ml) was used as a positive control for the experimental set-up. (B) Phosphorylation of endogenously expressed RET in PC6-3 cells. RET was immunoprecipitated (IP) with two different antibodies (C-19 and C-20). In the top panel the samples are stained with antibodies to phosphotyrosine, and in the middle and bottom panels the same filter was re-probed with the two different antibodies to RET. The cells were treated with full-length soluble GFR α 1 (1000 ng/ml) in the absence or presence of GDNF (100 ng/ml). WB, Western blot.

at a concentration of approx. 100 ng/ml, whereas GFR α 1¹⁴⁵ and GFR α 1¹¹⁴ mediated the same level of phosphorylation only at concentrations of approx. 500 ng/ml and 1000 ng/ml respectively (Figure 5B). The experiment was repeated three times with different batches of purified receptors. Despite some variations in phosphorylation levels, all three assays indicated that the truncated receptors were less active than full-length GFR α 1.

We also demonstrated that the use of RET-phosphorylation assays for the detection of small functional differences between GFR α 1 receptors is hampered by high concentrations of the receptors. In this experiment, 1000 ng/ml GFR α 1²⁰, GFR α 1¹¹⁴ or GFR α 1¹⁴⁵ was applied. Under these conditions, the differences in their capacity to mediate the phosphorylation of RET were difficult to visualize (Figure 5C). The reason why the neurite outgrowth assay is more sensitive than the phosphorylation assay, in spite of high concentrations of soluble GFR α 1, remains unclear. However, the neurites were counted after 3–4 days, and the concentrations of GDNF and GFR α 1 may fall during the long incubation time.

DISCUSSION

The GDNF-binding receptor GFR α 1 is attached to the membrane by a GPI anchor and consists of three similar cysteine-rich do-

Figure 5 RET-phosphorylation assays

(A) GPI-anchored receptors in MG87-RET cells. Transiently expressed GFR α 1¹²² induced equally strong phosphorylation of RET as did GFR α 1^{WT}. Top panel, samples stained with antibodies to phosphotyrosine; middle panel, filters re-probed with antibodies to RET; bottom panel, demonstration of equal expression levels of the two GPI-anchored receptors by staining with anti-FLAG antibodies. The samples for the bottom panel were taken before immunoprecipitating with antibodies to RET. (B) Effects of decreasing concentrations of soluble GFR α 1 in MG87-RET cells. The soluble receptor variants GFR α 1¹¹⁴, GFR α 1¹⁴⁵ and GFR α 1²⁰ are indicated on the right. (C) High concentrations of soluble GFR α 1. At 1000 ng/ml, truncated soluble GFR α 1¹¹⁴ and GFR α 1¹⁴⁵ induced equally strong phosphorylation of RET as did full-length GFR α 1²⁰. In the first two lanes no soluble GFR α 1 was added, to show that GDNF alone in this cell line does not induce any background phosphorylation of RET. IP, immunoprecipitation; WB, Western blot.

main. The region corresponding to D2 and D3 has been shown previously to participate in ligand binding as well as to interact with RET [10,11]. So far the function of D1 has remained unclear. In the present work we used three different methods to characterize the function of D1 of GFR α 1. The first of these was direct binding assays between GDNF and different forms of GFR α 1 (full length and truncated). These assays showed that the presence of D1 stabilizes the binding of GDNF to both the soluble

and the GPI-anchored receptor. Moreover, cell-free binding assays with purified GFR α 1 variants showed that D1 fulfils this function independently of other components such as RET, heparan sulphates [26] and N-CAM [27]. The second method was neurite outgrowth assays with soluble GFR α 1 variants, which showed that truncated versions of GFR α 1 were biologically less active. The third method was RET-phosphorylation assays, which distinguished between the full-length and truncated receptors only at low GFR α 1 concentrations. Taken together, our results show that D1 of GFR α 1 is important for stabilizing the interaction between GFR α 1 and its ligand GDNF.

The reason why earlier studies failed to detect this function of D1 probably lies in the experimental set-up. First, when GFR α 1 is overexpressed in a cell for a phosphorylation assay, the amount of RET might become rate limiting. Even if only part of the GFR α 1 protein binds GDNF, the amount of GDNF-bound GFR α 1 might still be high enough to activate all available RET. This seemed to be the case in our own phosphorylation assays, both with overexpressed GPI-anchored GFR α 1 and with high concentrations of soluble GFR α 1. Secondly, if high levels of GDNF, which exceed or are close to the determined K_d values, are used for cross-linking experiments, most GFR α 1 receptors will become saturated. If this is the case, slight differences in K_d values may become difficult to detect. In our cell-based binding assays and SPAs, the IC_{50} values for the interaction of GDNF with full-length GFR α 1 were 1.12 ± 0.14 nM and 0.75 ± 0.15 nM respectively. In these assays, the concentration of ^{125}I -GDNF was much lower than the obtained IC_{50} values, which therefore correspond approximately to K_d values. The K_d values determined for the GDNF–GFR α 1 complex using different binding assays vary in the literature from 2.3 pM [23] and 63 pM [20] to 0.9 nM [11] and 1.9 nM [25], whereas cross-linking experiments usually are carried out with 1 nM iodinated GDNF.

The activation of RET is thought to be dependent on its dimerization and subsequent transphosphorylation [29]. The current view on how this dimerization occurs is based on the well documented formation of GDNF homodimers [30]. According to this view, dimeric GDNF binds to two GPI-anchored GFR α 1 proteins and triggers the formation of a tetrameric GDNF–GFR α 1 complex. Although such a complex has never been well documented experimentally, it is thought to bring two RET molecules together on the extracellular side of the plasma membrane [11,23]. The dimerization of RET^{ED} leads to the dimerization of the intracellular tyrosine kinase domains of RET as well, which then transphosphorylate each other and thereby trigger the intracellular signalling cascade. The results from our binding assays show that D1 stabilizes the interaction between GDNF and GFR α 1, but does not seem to participate in the interaction with RET. Given that the receptor complex consists of a central dimer of GDNF, which symmetrically binds two GFR α 1 molecules, there are several possibilities as to how D1 could stabilize the complex.

One way in which D1 could stabilize the complex is by a direct D1–D1 interaction. This would stabilize the dimer and, via that, the GDNF–GFR α 1 complex. However, as a D1-based dimerization of GFR α 1 could also lead to the formation of dimers in the absence of GDNF, a direct D1–D1 interaction would need to be very weak in order not to cause the self-activation of RET in the absence of GDNF. In our gel filtration assays both forms of GFR α 1 eluted consistently with their monomeric molecular mass, and a direct D1–D1 interaction therefore seems unlikely. Another possibility is a direct interaction between D1 and GDNF. Although the major binding site of GDNF is in the cleft between D2 and D3 [11], D1 might ‘reach down’ to GDNF by bending the potential hinge, consisting of approx. 30 amino acid residues [7,11]. A third possibility is that D1 could interact both with GDNF

and a subdomain of GFR α 1. Previously published cross-linking experiments with chimaeric and truncated GFR α 1 and GFR α 2 forms [10] showed that D1 was dispensable. In spite of this main conclusion, the authors noted that some of the deletion mutants cross-linked GDNF with lower efficiency than the full-length receptor. Our own cross-linking experiments (results not shown) supported these findings. Further studies, such as crystallization of the GDNF–GFR α 1 complex, are clearly needed to establish in detail how D1 stabilizes the binding of GDNF to GFR α 1.

Compared with the major binding sites for RET and GDNF on D2 and D3 of GFR α 1, D1 has a minor but important stabilizing and optimization role in the function of GFR α 1. It is clear that not all biological processes are governed by large changes. Indeed, modest changes in expression levels or enzymatic activities have been reported previously to have clear physiological implications, which even may underlie diseases [31,32]. The stabilizing effect of D1 may become important under conditions where the concentration of the ligand is restricted, and the spreading of GDNF in tissues has indeed been shown to be a ‘bottleneck’ in therapeutic applications [33]. The impact of the interaction between D1 of GFR α 1 and GDNF should therefore be taken into account when designing GDNF analogues for therapeutic use.

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