Determinants of Ligand Binding Specificity in the Glial Cell Line-derived Neurotrophic Factor Family Receptor α\(a\)*

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The glial cell line-derived neurotrophic factor (GDNF) family comprise a subclass of cystine-knot superfamily ligands that interact with a multisubunit receptor complex formed by the c-Ret tyrosine kinase and a cysteine-rich glycosyl phosphatidylinositol-anchored binding subunit called GDNF family receptor α (GFRα). All four GDNF family ligands utilize c-Ret as a common signaling receptor, whereas specificity is conferred by differential binding to four distinct GFRα homologues. To understand how the different GFRαs discriminate ligands, we have constructed a large set of chimeric and truncated receptors and analyzed their ligand binding and signaling capabilities. The major determinant of ligand binding was found in the most conserved region of the molecule, a central domain predicted to contain four conserved α helices and two β strands. Distinct hydrophobic and positively charged residues in this central region were required for binding of GFRα1 to GDNF. Interaction of GFRα1 and GFRα2 with GDNF and neurturin required distinct subsegments within this central domain, which allowed the construction of chimeric receptors that responded equally well to both ligands. C-terminal segments adjacent to the central domain are necessary and have modulatory function in ligand binding. In contrast, the N-terminal domain was dispensable without compromising ligand binding specificity. Ligand-independent interaction with c-Ret also resides in the central domain of GFRα1, albeit within a distinct and smaller region than that required for ligand binding. Our results indicate that the central region of this class of receptors constitutes a novel binding domain for cystine-knot superfamily ligands.

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1 The abbreviations used are: GDNF, glial cell line-derived neurotrophic factor; NTN, neurturin; PSP, persephin; ART, artemin; GPI, glycosyl phosphatidylinositol; GFRα, GDNF family receptor α; HA, hemagglutinin.

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Determinants of Ligand Binding in GFRα Receptors

autonomous and non-cell-autonomous functions have been proposed for GFRα receptors expressed in the absence of c-Ret. Recent evidence obtained in c-Ret-deficient cell lines and primary sensory neurons isolated from c-Ret knock-out mice indicates the existence of alternative signaling mechanisms mediated by GFRα receptors acting in a cell-autonomous manner independently of c-Ret (23, 24). GFRα receptors may also function in a non-cell-autonomous manner to capture and concentrate diffusible GDNF family ligands from the extracellular space and then present these factors in trans to afferent c-Ret-expressing cells (25, 26).

In the work presented here, we have explored the molecular basis of ligand recognition by GFRα receptors. A large collection of chimeric and truncated receptors was generated and tested in binding and c-Ret autoprophosphorylation assays. The results of these experiments allowed us to identify a determinant of ligand binding specificity in a central region containing four predicted α helices and two short β strands.

**EXPERIMENTAL PROCEDURES**

**Reagents—**The cDNAs for rat GFRα1, rat GFRα2, and mouse GFRα3 subunits were subcloned in the pCDNA3 vector (Invitrogen) as described previously (21). The cDNA for chicken GFRα1 (27), a gift from Alun Davies (University of Edinburgh, Edinburgh, United Kingdom), was also subcloned in pCDNA3. Monoclonal IgG against hemagglutinin was from BabCO; anti-phosphotyrosine monoclonal IgG, PY99, and goat polyclonal antibodies, C-20 and T-20, against c-Ret were from Santa Cruz Biotechnology. GDNF was prepared in Sf21 cells as described previously (28). NTN was from Peprotech. Recombinant ART4 (27), a gift from Jannssen Research Foundation (Belgium), was from R&D Systems. Reagents for radiiodination and cross-linkings were purchased from Amersham Pharmacia Biotech, Sigma, and Pierce.

**Construction of Chimeric Receptors and Deletions—**An hemagglutinin (HA) epitope (YPYDVPDYA) was inserted after the putative signal peptide sequence of each GFRα construct by Kunkel mutagenesis (29). Both Kunkel method and QuickChange mutagenesis (Stratagene) were used to incorporate novel restriction sites at selected conserved strings of residues PYE (BsiWI), RRR (NarI), and SGN (BspEI). Binary chimeras were prepared using these new restriction sites for segmental exchanges. Each exchange site was located in predicted loop regions in the different receptor constructs. Secondary structure predictions were done using PHD Predict (30). Alignments were done with Clustal X. Cross-linking Assay—GFRα receptors acting in a cell-autonomous manner independently of c-Ret (23, 24). GFRα receptors may also function in a non-cell-autonomous manner to capture and concentrate diffusible GDNF family ligands from the extracellular space and then present these factors in trans to afferent c-Ret-expressing cells (25, 26).

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**Cross-linking Assay—**Ligands were radiiodinated to specific activities of about 0.5 to 2 × 10⁶cpm/μg by the lactoperoxidase method as described previously (28). COS cells were transfected using DEAE-dextran. 48 h after transfection, cells were rinsed once with chilled binding buffer (1 mg/ml bovine serum albumin, 1 mg/ml glucose, 0.1 mM CaCl₂, 0.1 mM MgCl₂ in phosphate-buffered saline) and equilibrated with binding buffer containing 50–100 ng/ml radiiodinated ligand for 4 h at 4 °C. Subsequently, bis(succinimidyl) suberate (BS3) cross-linker was added to a final concentration of 0.8 mM and incubated for an additional 4 h at 4 °C. The reaction was quenched by adding 50 mM glycine. Cells were rinsed twice with chilled 50 mM glycine in phosphate-buffered saline and lysed in RIPA buffer. Aliquots were taken from each lysate for HA immunoblot lower panels (Fig. 1A, lower panels) was used to normalize the binding to the levels of expression of the different GFRα receptors for quantification. These experiments confirmed the main established interactions between GDNF family ligands and GFRα receptors (Fig. 1B, solid arrows) and revealed a number of additional interactions of lower affinity between non-cognate pairs (Fig. 1B, dotted arrows). Interestingly, although GFRα3 was only able to bind ART, this ligand was capable of interacting, to varying degrees, with all GFRα receptors (Fig. 1A). Conversely, although PSP binding could only be detected to GFRα4, this receptor was able to interact with lower affinity with all members of the GDNF ligand family (Fig. 1A).

**Domain Boundaries and Chimeric Receptors—**Secondary structure predictions indicated that GFRα receptors contain primarily α helices connected by shorter segments of undefined structure, presumably representing loops (Fig. 2). Two predicted short β strands are also conserved in the central region of all four GFRαs (Fig. 2). The remarkable conservation of the predicted pattern of secondary structure elements among different GFRα receptors suggests that these may represent true structural elements in this class of receptors. For the first set of chimeric receptors, we initially defined two internal boundaries corresponding to two highly conserved triplets in the primary sequence of GFRα receptors, i.e., PYE and SGN (Fig. 2). These boundaries coincide with interhelical regions in secondary structure predictions and divide the GFRα molecule into N-terminal (three predicted helices), central (four predicted helices and two β strands), and C-terminal (two predicted helices, 30-40 residues) domains. Similar domain boundaries have also been defined by others (5). The percentage similarity of each of the domains in GFRα2, -3, and -4 with respect to GFRα1 is shown in Table 1. Unique restriction sites, BsiWI and BspEI, were created by silent mutagenesis at the conserved PYE and SGN triplets, respectively, in all four GFRα receptors. Chimeric molecules were then

**Phosphorylation and GFRα-Ret Interaction Assays—**Neuro2A cells were transfected with the different GFRα constructs by the calcium phosphate precipitation method. The day after transfection, cells were switched to serum-free medium containing 5 μM all-trans retinoic acid, 0.1% bovine serum albumin, and N2 supplements and were incubated for 16–20 h. An hour before stimulation, cells were incubated with fresh serum-free medium. Stimulations were done by adding ligands to the final concentration of 50 ng/ml for 12 min, after which cells were lysed in RIPA buffer. Aliquots were taken from each lysate for HA immunoblots on polyvinylidene fluoride membranes (Millipore) and detected using a chemiluminescence (Amersham Pharmacia Biotech). Both immunoblot and autoradiographic exposures were detected and quantified using a Storm840 phosphorfluorimager (Molecular Dynamics).
constructed by exchanging homologous domains using these two restriction sites.

The N-terminal Domain Is Not a Determinant of Ligand Binding Specificity—We constructed chimeras between two relatively distant members of the GFRα receptor family, GFRα1 and GFRα3, and tested their ability to bind 125I-labeled GDNF and ART and to activate the c-Ret receptor. These experiments indicated that the N-terminal domain is dispensable for ligand binding specificity. A receptor molecule containing the N terminus of GFRα3 and the central and C-terminal domains of GFRα1 (termed 3–1-1) was able to bind GDNF as efficiently as wild type GFRα1 (Fig. 3 A). Similarly, the 1–3-3 chimera was able to bind ART, albeit somewhat less efficiently than wild type GFRα3 (Fig. 3 A). On the other hand, exchanges involving the central and C-terminal domains disrupted ligand binding (Fig. 3 A), suggesting the requirement of

FIG. 1. Specificity and promiscuity in the interaction of GFRα receptors with GDNF family ligands. A, affinity labeling of GFRα receptors expressed in COS cells with iodinated GDNF family ligands. The lower panels show aliquots of cell lysates analyzed with HA antibodies by Western blotting. Numbers below the lanes indicate relative binding normalized to expression levels. Control (no GFRα receptor) was set to zero, whereas the cognate interaction was set to one. IB, immunoblot. B, the diagram shows the main (cognate) interactions between GDNF ligands and GFRα receptors (thick arrows), as well as different cross-reactivities (dashed arrows) revealed by affinity labeling experiments.

FIG. 2. Conservation of predicted secondary structure elements and domain boundaries in GFRα receptors. The aligned amino acid sequences of rat GFRα1, rat GFRα2, mouse GFRα3, and chicken GFRα4 are shown. Sequences predicted to correspond to α helices are bolded in red, and those to β strands in green. Signal peptide sequences are underlined at the N termini. Conserved triplets used for silent mutagenesis (PYE, RRR, and SGN) are bolded in black, and the introduced restriction sites are indicated with open arrows. Solid curved arrows denote the boundaries of the N- and C-terminal truncations made in GFRα1. All C-terminal truncations of GFRα1 retain the GPI signal peptide (underlined at the C termini) and the preceding 9 residues as indicated by the last solid curved arrow on the GFRα1 sequence. The boundaries of the ΔC21 deletion in GFRα1 are indicated with open curved arrows. Putative GPI cleavage sites are double underlined.
these domains for ligand interaction. The ability of the receptor chimeras to induce ligand-dependent autophosphorylation of c-Ret was examined after transient transfection into the neuroblastoma line Neuro2A, which expresses c-Ret endogenously. In agreement with the results from cross-linking experiments, only GFRα molecules retaining the central and C-terminal domains of GFRα1 were able to support GDNF-dependent stimulation of c-Ret tyrosine phosphorylation (Fig. 3B).

The results from chimeras between GFRα1 and GFRα3 receptors suggested that the N-terminal domain was not essential for ligand recognition and indicated the importance of the central and C-terminal segments of the GFRα molecule. To determine the generality of these observations for other members of the GFRα family, we constructed chimeras between GFRα2 and GFRα3 and tested their ability to bind 125I-labeled NTN and to activate the c-Ret receptor. The only molecule capable of significant NTN binding, in addition to wild type GFRα2, was the 3–2-2 chimera (Fig. 3C). This result indicated that, similar to GFRα1, the N terminus of GFRα2 is dispensable for ligand binding specificity, and both the central and C-terminal domains are required for interaction with NTN. In agreement with the cross-linking data, the 3–2–2 chimera also supported NTN-dependent c-Ret phosphorylation (Fig. 3D).

In Neuro2A cells, however, the 2–2–3 chimera was also able to mediate ligand-dependent c-Ret phosphorylation, despite its undetectable binding to 125I-NTN in COS cells (Fig. 3, C and D). These data are in accordance with previous observations indicating that c-Ret and GFRα receptors collaborate in the binding of suboptimal ligands and suggested that, under certain circumstances and in the presence of c-Ret, the C-terminal domain of GFRα receptors may also be exchanged without compromising ligand binding specificity (see below).

**The Central Domain Is a Crucial Determinant of Ligand Binding Specificity**—We then analyzed chimeras between more closely related receptors, i.e. GFRα1 and -2, GFRα1 and -4, and GFRα2 and -4. The chimeras between GFRα1 and GFRα2 revealed a reciprocal pattern of binding to 125I-GDNF and 125I-NTN (Fig. 4A). In all cases, the receptor chimeras showing the strongest binding to a given ligand were those retaining the central domain from the cognate receptor (Fig. 4, A and B). A number of GFRα molecules that showed low or undetectable binding, however, did mediate significant levels of c-Ret phosphorylation after GDNF stimulation, such as the 2–2-1 and the 2–2–2 molecules (Fig. 4C). Interestingly, all chimeras between GFRα1 and GFRα2, as well as wild type GFRα1, supported some degree of c-Ret activation after NTN stimulation (Fig. 4C, lower panels). These data are in agreement with results from previous studies demonstrating significant cross-reactivity between the GFRα1-GDNF and GFRα2-NTN systems in the presence of c-Ret (20, 21, 31) and support a role for c-Ret in modulating the interaction of the receptor complex with suboptimal ligands.

**Binding Determinants for GDNF and NTN Reside in Discrete Subcentral Domains of GFRα1 and GFRα2**—To further investigate structure-function relationships in the GFRα receptor family, we constructed chimeras involving subsegments of the central domain of GFRα1 and GFRα2 receptors. We took advantage of the conserved RRR triplet sequence located in the middle of the central domain, between the two predicted β strands (Fig. 2), to introduce a unique NarI site by silent mutagenesis. This new boundary subdivides the central domain into two roughly equal halves of approximately 100 residues, each predicted to contain two α helices and a β strand. Cross-linking studies with 125I-GDNF indicated that the chimeras that retained binding were the ones that contained the second central subsegment from GFRα1, i.e. corresponding to the formula X-2-1’-X (Fig. 5A). Conversely, the chimeras that retained binding to 125I-NTN contained the first central subsegment from GFRα2 (Fig. 5A), suggesting different structural requirements for ligand binding specificity within the central domains of GFRα1 and GFRα2. Thus, the chimeric receptor 2–2-1’-1, which combined both elements, had a broader specificity and was able to bind 125I-GDNF and 125I-NTN with comparable efficiency (Fig. 5A). As with the previous chimeras between these two receptors, all constructs supported some level of c-Ret phosphorylation over control when introduced into Neuro2A cells (Fig. 5B), although the molecules that bound ligand more efficiently were still the most active. This again confirms the contribution of c-Ret in ligand recognition and demonstrates that the low ligand binding efficiency of some of the chimeras was not because of structural problems in these molecules, as they were still able to support some degree of ligand-dependent c-Ret activation.

**Truncated GFRα Receptors Lacking the N-terminal Domain Retain Ligand Binding**—Although the N-terminal domain of GFRα receptors did not appear to play a role in ligand specificity, it was unclear whether it was at all necessary for ligand binding or it had some other function, such as contacting the c-Ret receptor. This question became all the more important in view of the proposed existence of a natural splice variant of GFRα2 lacking the first 146 residues (termed GFRα2c) (32). We therefore generated truncated versions of GFRα1 lacking the first (GFRα1-ΔN57), the first and the second (GFRα1-ΔN87), or all three (GFRα1-ΔN113) predicted α helices of the N-terminal domain (see Fig. 2). In addition, we also generated a GFRα2 construct corresponding to the reported GFRα2c splice variant, an analogous construct based on the GFRα1 receptor (GFRα1-ΔN144), and a GFRα1 construct lacking all first four predicted α helices, including predicted helix 4 in the first portion of the central domain that was essential for ligand binding (GFRα1-ΔN161) (see Fig. 2). All constructs carried an HA tag at the N terminus and were analyzed in cross-linking and c-Ret phosphorylation assays as above. The GFRα1-ΔN87 variant that lacked the first two predicted helices of the N terminus was not produced in COS cells, presumably because the deletion removed an odd number of cystines, which could have resulted in misfolding of the protein. On the other hand, all remaining

<table>
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<th>TABLE I</th>
<th>Percentage similarity of N-terminal (less signal sequence), central (PYE to SGN), and C-terminal (until GPI consensus sequence) domains of GFRα2 (rat), GFRα3 (mouse), and GFRα4 (chicken) receptors relative to GFRα1 (rat)</th>
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<tbody>
<tr>
<td>GFRα2</td>
<td>63.8</td>
</tr>
<tr>
<td>GFRα3</td>
<td>37.8</td>
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<tr>
<td>GFRα4</td>
<td>61.5</td>
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N-terminally truncated variants of GFRα1 (i.e. ΔN57, ΔN113, ΔN144, and ΔN161) were produced at normal levels (Fig. 6, A and B). The GFRα1 deletion mutants ΔN57, ΔN113, and ΔN144 were all able to bind GDNF, albeit with lower efficiency than the wild type receptor, particularly the ΔN57 deletion (Fig. 6A). The ΔN161 deletion construct was however unable to bind GDNF (Fig. 6B), in agreement with the importance of the central region of GFRα receptors for ligand binding. The GFRα2c splice variant (GFRα2-ΔN146) was able to bind 125I-NTN at a level comparable with that of wild type GFRα2 (Fig. 6C). Together, these data demonstrate that the N-terminal domain of GFRα receptors is not absolutely required for ligand binding, and, in the case of GFRα2, an equally efficient interaction can also take place in its absence.

To investigate whether portions of the C-terminal tail of GFRα1 may also be dispensable, we constructed a series of

**Fig. 3.** The N-terminal domain is not a determinant of ligand binding specificity. A, affinity labeling of chimeras between GFRα1 and GFRα3 receptors with 125I-GDNF and 125I-ART as indicated. The lower panels show aliquots of cell lysates analyzed with HA antibodies by Western blotting. Numbers below the lanes indicate relative binding normalized to expression levels. Control (no GFRα receptor) was set to zero, whereas the interaction with the preferred wild type receptor (i.e. 1–1-1 for GDNF and 3–3-3 for ART) was set to one. IB, immunoblot. B, phosphorylation of c-Ret induced by GDNF in Neuro2A cells expressing chimeras between GFRα1 and GFRα3 receptors. The middle panel shows reprobing of the same filter with anti-c-Ret antibodies. The lower panel shows aliquots of cell lysates analyzed with HA antibodies. C, affinity labeling of chimeras between GFRα2 and GFRα3 receptors with 125I-NTN. D, phosphorylation of c-Ret induced by NTN in Neuro2A cells expressing chimeras between GFRα2 and GFRα3 receptors. E, summary of results.

**Fig. 4.** The central domain is a crucial determinant of ligand binding specificity. A, affinity labeling of chimeras between GFRα1 and GFRα3 receptors with 125I-GDNF and 125I-NTN as indicated. The lower panels show aliquots of cell lysates analyzed with HA antibodies by Western blotting. Numbers below the lanes indicate relative binding normalized to expression levels. Control (no GFRα receptor) was set to zero, whereas the interaction with the preferred wild type receptor (i.e. 1–1-1 for GDNF and 2–2-2 for NTN) was set to one. IB, immunoblot. B, affinity labeling of chimeras between GFRα1 and GFRα4 receptors with 125I-GDNF. C, phosphorylation of c-Ret induced by GDNF and NTN in Neuro2A cells expressing chimeras between GFRα1 and GFRα3 receptors. The middle panel in each set shows reprobing of the corresponding filter with anti-c-Ret antibodies. The lower panels show aliquots of cell lysates analyzed with HA antibodies. D, phosphorylation of c-Ret induced by GDNF in Neuro2A cells expressing chimeras between GFRα1 and GFRα4 receptors. E, summary of results.
C-terminal truncations (see Fig. 2) upstream of Gly-421 and the putative GPI anchor signal sequence (last black arrow in Fig. 2). All three GFRα1 C-terminal deletion constructs were produced in COS cells, and both DC31 and DC55 retained their ability to bind 125I-GDNF (Fig. 6B). A compound deletion mutant lacking 144 residues in the N terminus and 31 residues in the C terminus (GFRα1-DN144-DC31) was also able to bind GDNF (Fig. 6B). However, the ΔC79 deletion, which disrupts a predicted helix in the C-terminal domain of GFRα1 (see Fig. 2), was unable to bind GDNF (Fig. 6B). The region immediately downstream of this predicted helix does not appear to be necessary for GDNF binding, as indicated by the activity of the ΔC21 deletion (Fig. 6D). Together, these data indicate that, in the C-terminal region of GFRα receptors, the integrity of the predicted helices is required for ligand binding.

The N-terminal Domain of GFRα Receptors Is Dispensable for Ligand-dependent or -independent Interactions with c-Ret—The question whether the N- and C-terminal domains of GFRα receptors play any role in the interaction with c-Ret was addressed in several ways. To investigate ligand-dependent interactions, c-Ret autophosphorylation assays were performed in Neuro2A cells carrying truncated GFRα receptors. In agreement with the binding data, the DN57, DN113, and DN144 GFRα1 truncated receptors were all able to induce GDNF-dependent stimulation of c-Ret phosphorylation (Fig. 7, A and B), suggesting that the N-terminal domain of GFRα1 is not crucial for the contact of the GFRα1-GDNF complex with c-Ret. Also in agreement with their ability to cross-link 125I-GDNF, the DC31 and DC55 C-terminal deletion mutants, as well as the DN144ΔC31 compound mutant, also mediated ligand-dependent stimulation of c-Ret phosphorylation (Fig. 7, B and C). Likewise, the GFRα2c splice variant, lacking the first 146
N-terminal residues, was also able to mediate NTN-dependent c-Ret activation (Fig. 7D). As expected, the truncated GFRα1 receptors that did not bind GDNF (i.e. ΔN161 and ΔC79) were not able to stimulate c-Ret phosphorylation (Fig. 7C).

We also determined whether the N- and C-terminal regions of GFRα1 were required for ligand-independent interaction with the c-Ret receptor using a co-immunoprecipitation assay in transiently transfected COS cells, which do not produce detectable levels of GDNF. Care was taken to solubilize membrane lipid rafts so as to assure that co-immunoprecipitation of GFRα1 and c-Ret reflected direct interaction and not co-existence in the same membrane compartment (see “Experimental Procedures”). Immunoprecipitation of c-Ret brought down HA-tagged wild type GFRα1 receptors only in cells that received the c-Ret expression plasmid, indicating a direct receptor-receptor interaction in the absence of ligand (data not shown). All N- and C-terminal deletions of GFRα1 were able to interact with c-Ret in this assay, suggesting that these regions are not involved in the interaction of this receptor with c-Ret (data not shown). Intriguingly, also the GFRα1-ΔN161 and ΔC79 deletion mutants, which were unable to bind GDNF and to activate c-Ret, were still capable of interacting with the c-Ret receptor in a ligand-independent manner (data not shown), indicating that distinct structural determinants in GFRα1 may be required for ligand binding and association with c-Ret, respectively.

Distinct Hydrophobic and Positively Charged Residues in the Central Domain of GFRα1 Mediate Binding to GDNF—To begin to identify individual amino acid residues in GFRα1 involved in the binding of GDNF, we searched for short stretches of residues in the central domain (predicted helices 4 to 7) with chemical properties complementary to the GDNF binding surface. Previous site-directed mutagenesis studies have identified sets of hydrophobic and negatively charged residues in exposed loop regions of GDNF that are required for efficient binding to GFRα1 (22). Two motifs in the center of the GFRα1 molecule, the hydrophobic triplet MLF in the first predicted β strand (β1 in Fig. 2) and the basic triplet RRR (NarI site in Fig. 2), were selected for site-directed mutagenesis and functional analysis. Replacement of MLF into an alanine triplet (α1 MLF-A3) abolished binding of GFRα1 to GDNF (Fig. 8A). Moreover, replacement of RRR into an alanine triplet (α1 R3-A3) also impaired the ability of GFRα1 to interact with GDNF (Fig. 8A). Thus, distinct hydrophobic and positively charged residues in the central domain of GFRα1 mediate binding to GDNF. Despite their inability to bind GDNF, however, both the MLF-A3 and R3-A3 GFRα1 mutants retained the capacity of mediating substantial levels of c-Ret phosphorylation in the presence of GDNF (Fig. 8B), indicating that they do not form part of the interaction site to c-Ret.

**DISCUSSION**

In this study, we have investigated the structural determinants required for ligand binding specificity in the family of GFRα receptors. This class of receptors has a unique pattern of cystine residues, predicted α helices, β strands, and loops spread over approximately 400 amino acid residues and lack many of the domains most commonly present in other receptors, such as leucine repeats, immunoglobulin, and fibronectin-like domains. Thus, the GFRα receptor family likely represents a structurally novel receptor class. Among the four members of this family, the central region of the molecule between the PYE and SGN motifs shows the highest conservation, whereas the N-terminal and, in particular, the C-terminal regions are much
Determinants of Ligand Binding in GFRα Receptors

The major finding of our study is the localization of the ligand binding specificity domain of GFRα receptors to the central region of the molecule containing four predicted α helices and two short β strands (Fig. 8C). The localization of the determinant of ligand specificity in GFRα receptors to the most conserved part of the molecule, as opposed to the most variable N- and C termini, was unexpected. The fact that the C-terminal domains of GFRα-1, -2, or -4 could not be substituted for that of GFRα-3 without loss of ligand binding is in agreement with the latter being the most divergent member of the GFRα family. Although a portion of this domain could be deleted without loss of ligand binding, the integrity of the two predicted α helices present in this region could not be compromised. Together, these data suggest that some determinants in the C-terminal part of the GFRα molecule may also contribute to ligand binding. In contrast, the N-terminal region could be exchanged among all receptors without loss of binding. In GFRα-1 and -2, this region could be deleted without abolishing ligand interaction, indicating that it does not significantly contribute to ligand binding. Our results also indicate that the N-terminal domain of GFRα receptors does not participate in the recruitment and activation of the c-Ret molecule, nor in ligand-independent interactions between the two receptors. Preliminary structural analysis of GFRα-1 by cyanogen bromide digestion and Edman microsequencing indicates that the central region that was most relevant to ligand binding in GFRα-1 forms a core structural unit reinforced by disulfide bridges. No disulfide bridges appear to link this core with the N-terminal region, suggesting that the latter forms a distinct structural domain, separated from the rest of the GFRα molecule. Interestingly, the putative mammalian homologue of the chicken GFRα-4 has recently been isolated and shown to lack the N-terminal domain altogether (33, 34). In agreement with our findings, this molecule is still capable of binding PSP (34). Thus, at least two GFRα receptor variants, the GFRα-2c splice variant and the mammalian GFRα-4 homologue, have lost the N-terminal domain without any apparent loss of function, suggesting that this region of the molecule may not be under a strong evolutionary pressure.

It is possible that the N-terminal domain of GFRα receptors plays other roles distinct from ligand binding. Several ligand-dependent signaling events have been described in cells expressing GFRα receptors in the absence of c-Ret (23, 24), presumably mediated by the collaboration of GFRα receptors with other transmembrane proteins. Thus, it is possible that the N-terminal domain of GFRα receptors plays a role in the interaction with transmembrane molecules other than c-Ret. Another possibility is suggested by a recent study indicating that N-glycans, as opposed to the GPI anchor itself, mediate the apical sorting of GPI-anchored receptors in epithelial cells (35). It is possible that the N-terminal domain of GFRα receptors participates in the polarized sorting of the receptor in neurons and that alternative splicing of this domain regulates the targeting of receptor molecules to different subcellular compartments.

Two sets of residues were identified as critical for the interaction of GFRα-1 with GDNF. The MLF and RRR triplets in the central region of GFRα-1 have complementary properties to the receptor binding surface identified in GDNF, characterized by hydrophobic and negatively charged residues (22). In GDNF, Ile-64, Leu-114, Leu-118, Tyr-120, and Ile-122 form a hydrophobic patch, whereas Asp-52, Glu-61, Glu-62, and Asp-116 form a negatively charged patch, which are both required for binding to GFRα-1, suggesting that they could be interacting with the MLF and RRR triplets, respectively, that we identified here in GFRα-1. Interestingly, the MLF and RRR sequences are highly conserved among members of the GFRα family (see Fig. 2), indicating that these residues do not represent specificity determinants but rather form part of a common epitope in GFRα receptors for binding to GDNF family ligands. In the neurotrophin family, the crystal structures of the ligand binding domains of Trk receptors (36) and of the TrkA-nerve growth factor complex (4), as well as extensive site-directed mutagenesis studies (37–40), bring support to the idea that specificity among related members of families of cognate ligands and receptors is provided by variable residues that modulate the affinity of a core binding interface that is common to all family members.

Interestingly, several GFRα chimeras, including the three point mutants made in GFRα-1 that showed no or little ligand binding, were still able to mediate ligand-dependent activation of c-Ret, indicating that suboptimal GFRα receptors can still contribute to the formation of a functional receptor complex in the presence of c-Ret. These data are in agreement with reciprocal observations made with several GDNF mutants and non-cognate ligand-receptor pairs showing that some ligands with low or negligible affinity for individual GFRα receptors may still be able to activate c-Ret in a GFRα-dependent manner (11, 22). Thus, either a residual affinity between suboptimal pairs of GDNF ligands and GFRα is still capable of recruiting c-Ret to stabilize the complex, or, as previously proposed, GFRα and c-Ret exist to some extent in a pre-formed complex that allows the interaction of suboptimal ligands or suboptimal GFRα molecules. In either case, our results indicate that c-Ret facilitates suboptimal interactions between GDNF family ligands and GFRα receptors and confirm the role of c-Ret in ligand recognition. These observations also indicate that structure-function studies of GDNF ligands and GFRα based only on functional responses without direct assessment of ligand-receptor binding are likely to miss the actual determinants directly involved in these interactions (see for example Ref. 41).

Another interesting observation made in the present study concerns the two deletion constructs of GFRα-1, i.e. ΔN161 and ΔC79, that were still capable of forming a complex with c-Ret in the absence of ligand, despite their complete inability to bind GDNF or mediate GDNF-dependent c-Ret phosphorylation. In fact, nearly half of the GFRα-1 molecule (161 residues from the N terminus and 79 residues from the C terminus) may be deleted without affecting its ability to interact with the c-Ret receptor. This result suggests that, although ligand and c-Ret binding require the same central domain in GFRα receptors, distinct structural determinants within this domain participate in its interaction with GDNF and c-Ret.

In conclusion, GFRα receptors utilize a relatively conserved, central region of the molecule for both ligand binding and interaction with c-Ret. Other parts of the GFRα molecule appear to play modulatory roles in ligand binding and c-Ret activation. The N terminus in particular may have novel functions, possibly in subcellular sorting or in the interaction of GFRα receptors with other transmembrane molecules.

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REFERENCES