THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 276, No. 2, Issue of January 12, pp. 1450–1458, 2001

Printed in U.S.A.

Determinants of Ligand Binding Specificity in the Glial Cell Line-derived Neurotrophic Factor Family Receptor αs*

Received for publication, July 12, 2000, and in revised form, September 26, 2000
Published, JBC Papers in Press, October 3, 2000, DOI 10.1074/jbc.M006157200

Rizaldy P. Scott and Carlos F. Ibáñez‡

From the Division of Molecular Neurobiology, Department of Neuroscience, Karolinska Institute, 17177 Stockholm, Sweden

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.

Binding of polypeptide growth factors to cell surface receptors is the first event in the series of protein-protein interactions leading to the distinct biological responses characteristic of a given factor. Understanding the molecular interactions underlying the affinity and selectivity of ligand-receptor complexes is of fundamental importance if we are to comprehend fully the molecular basis of signal transduction by polypeptide growth factors.

A large group of growth factors contain a distinctive structural motif, the "cystine knot," formed by a cluster of three disulfide bridges, with two disulfide bridges and their connecting residues forming a ring structure through which the third disulfide bridge passes (1, 2). The protomer of cystine-knot superfamily factors is typically elongated and formed primarily by β strands connected by highly flexible hairpin loops, where most of the sequence variability among paralog factors is located. Different configurations of dimer assembly lead to various shapes, elongated either along or across the 2-fold symmetry axis, which are characteristic of the different subfamilies. Two high-resolution structures of the complex of a cystine-knot factor with its corresponding receptor have been obtained recently, that of vascular endothelial growth factor with the ligand binding domain of Flt-1 (3) and the one of nerve growth factor with the ligand binding domain of TrkA (4). In both cases, distinct immunoglobulin-like domains in the receptors are both necessary and sufficient for ligand binding. However, not all receptors of cystine-knot superfamily ligands contain immunoglobulin-like domains, suggesting the existence of novel binding domains for this class of ligands.

GDNF[1] is the prototype member of a new subfamily of cystine-knot ligands with important roles in the control of neuron survival and differentiation (5), kidney morphogenesis (6), and sperm cell development (7). In vertebrates, the GDNF family is known to comprise the following four distinct proteins: GDNF (8), neurturin (NTN) (9), persephin (PSP) (10), and artemin (ART) (11), also identified as enovin (12) or neublastin (13). An unusual feature of the receptor complex for GDNF family ligands is the requirement of two receptor subunits, one specialized in ligand binding and another in transmembrane signaling. All four members of the GDNF family signal through activation of the c-Ret receptor tyrosine kinase (14–17). c-Ret, however, can not bind ligand on its own but requires the presence of a glycosyl phosphatidylinositol (GPI)-anchored co-receptor known as GDNF family receptor α (GFRα) (18, 19). Interestingly, four different GFRα receptors have been identified (GFRαs 1 to 4), each selective, although not totally specific, for one member of the GDNF ligand family (reviewed in Ref. 5).

Experiments using purified proteins (20) and overexpression in cell lines (21) have demonstrated that c-Ret and GFRα receptors can also associate with low-affinity in the absence of ligand. In the presence of c-Ret, the ligand binding specificity of GFRα receptors broadens, allowing them to interact with different GDNF family ligands, as well as several mutants with defective GFRα binding (11, 20–22). *In vivo*, GFRα receptors are more widely expressed than c-Ret, suggesting additional roles for these "ectopic" sites of GFRα expression. Both cell-

* Financial support was obtained from the Swedish Medical Research Council (K99–33X-10908–06C), the European Commission (BMH4–97-2157), the Göran Gustafssons Stiftelse, and the Karolinska Institute. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed: Div. of Molecular Neurobiology, Dept. of Neuroscience, Karolinska Inst., Berzelius väg 1, 17177 Stockholm, Sweden. Tel.: 46-8-728-7660; Fax: 46-8-33-9548; E-mail: carlos@cajal.mbb.ki.se.

[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.

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 autophosphorylation 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 subcloned in the pCDNA3 vector (Invitrogen) were obtained as described previously (21). The cDNA for chicken GFRα4 (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 ART and PSP were generous gifts from Jannssen Research Foundation (Belgium). GFRα1-Fc was from R&D Systems. Reagents for radioiodination 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 (*Bsi*WI), RRR (*Nar*I), and SGN (*Bsp*EI). 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. N-terminal deletions for GFRα1 (ΔN57, ΔN84, ΔN113, and ΔN144) and for GFRα2 (ΔN126) were obtained by polymerase chain reaction using upstream primers containing the HA epitope sequence facilitating the grafting of the appropriate signal peptide using *Nde*I sites within the epitope and the cytomegalovirus promoter region of pCDNA3 (see Fig. 2). C-terminal deletions in GFRα1 (ΔC31, ΔC55, and ΔC79) upstream of Gly-421 (to retain the GPI anchor) were made sequentially from the smallest to the largest truncation using QuickChange mutagenesis.

Cross-linking Assay—Ligands were radioiodinated to specific activities of about 0.5 to 2×10^8 cpm/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 $CaCl_2$, 0.1 mM $MgCl_2$ in phosphate-buffered saline) and equilibrated with binding buffer containing 50–100 ng/ml radioiodinated ligand for 4 h at 4 °C. Subsequently, bis(succinimidyl) suberate (BS^3) cross-linker was added to a final concentration of 0.8 mM and incubated for an additional 45 min 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 (50 mM Tris-HCl, pH 7.4, 50 mM NaCl, 50 mM NaF, 1% IGEPAL CA-630, 0.25% sodium deoxycholate, 10% glycerol, 1 mM EDTA, 10 mM 2-glycerolphosphate, 2 mM Na_3VO_4). To assess expression of the various GFRα constructs, an aliquot of each lysate was used for immunoblots on polyvinylidene difluoride (Hybond P) membrane processed for detection of the HA tag. The rest of the lysates were immunoprecipitated using the anti-HA antibody and run on a 10% SDS polyacrylamide gel. Immunoblot analysis was done using enhanced chemifluorescence (Amersham Pharmacia Biotech). Both immunoblot and autoradiographic exposures were detected and quantified using a Storm840 phosphor/fluorimager (Molecular Dynamics).

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 a final concentration of 50 ng/ml for 12 min, after which cells are lysed in RIPA buffer. Aliquots were taken from each lysate for HA immunoblot analysis, whereas the rest of the lysates were used for c-Ret immunoprecipitations. c-Ret phosphorylation was assessed by immunoblotting using an anti-phosphotyrosine antibody.

To assess ligand-independent association of different GFRα constructs with c-Ret, co-transfections were done in COS cells using DEAE-dextran. Transfected cells were lysed 48 h later in RIPA buffer containing 60 mM β-octylglucoside. The presence of both sodium deoxycholate and β-octylglucoside in the cell lysis buffer assured complete solubilization of membrane lipid rafts, which was required to establish the co-existence of both GFRα and c-Ret receptors in the same molecular complex, as opposed to the same subcellular compartment. Cell lysates were immunoprecipitated for c-Ret and analyzed by Western blotting with anti-HA antibodies, recognizing epitope-tagged GFRα constructs.

RESULTS

Specificity and Promiscuity in the Interaction of GFRα Receptors with GDNF Family Ligands—No previous study had examined the binding specificity of all four GDNF family ligands to all four members of the GFRα receptor family in the same experiment. We used ^{125}I-labeled GDNF family ligands in chemical cross-linking assays to COS fibroblasts transiently expressing different GFRα receptors carrying an HA tag at the N terminus (Fig. 1*A*). In all cases, affinity labeling of the receptor band could be prevented by addition of an excess (up to 100-fold) of the corresponding unlabeled ligand (data not shown), indicating specific binding. Western blotting of the same cell lysates with anti-HA antibodies (Fig. 1*A*, *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. 1*B*, *solid arrows*) and revealed a number of additional interactions of lower affinity between non-cognate pairs (Fig. 1*B*, *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. 1*A*). 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. 1*A*).

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) domains of roughly 100, 200, and 100 residues, respectively. 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 I. Unique restriction sites, *Bsi*WI and *Bsp*EI, were created by silent mutagenesis at the conserved PYE and SGN triplets, respectively, in all four GFRα receptors. Chimeric molecules were then

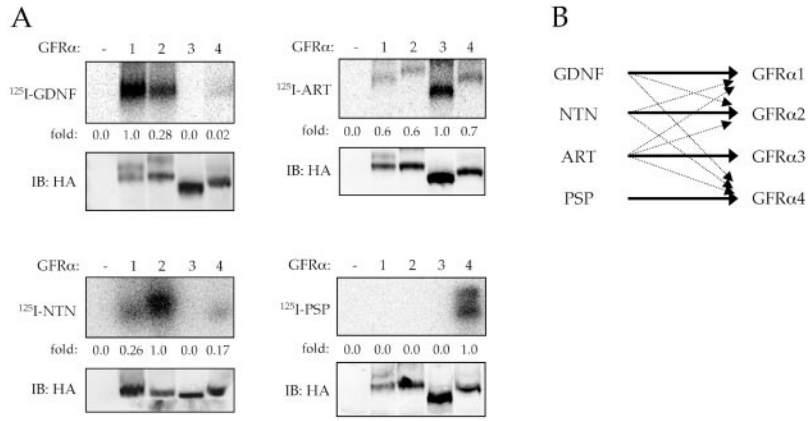

FIG. 1. **Specificity and promiscuity in the interaction of GFRα receptors with GDNF family ligands.** *A*, affinity labeling of GFRα receptors expressed in COS cells with iodinated GDNF family ligands. The *lower panels* show aliquots of cell lysates analyzed with HA antibodies by Western blotting. *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.

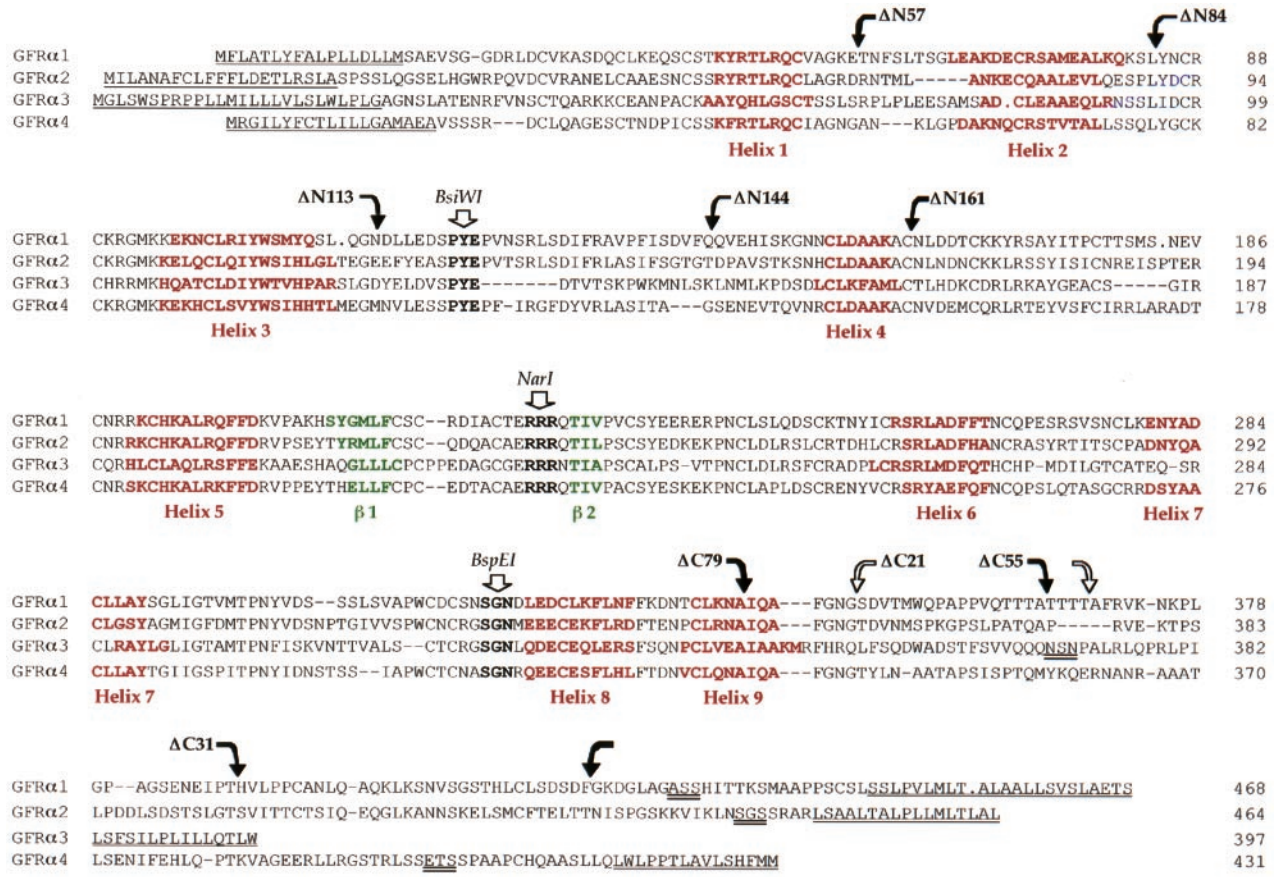

FIG. 2. **Conservation of predicted secondary structure elements and domain boundaries in GFRα receptors.** The aligned amino acid sequences of rat GFRα1, rat GFRα2, mouse GFRα3, and chicken GFRα4 are shown. Sequences predicted to correspond to α helices are *bolded* in *red*, and those to β strands 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*.

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 ^{125}I-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

TABLE I
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)

	N-terminal	PYE to SGN	C-terminal
GFRα2	63.8	65.7	48.6
GFRα3	37.8	43.9	34.7
GFRα4	61.5	64	47.3

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. 3*B*).

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 ^{125}I-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. 3*C*). 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. 3*D*). In Neuro2A cells, however, the 2–2-3 chimera was also able to mediate ligand-dependent c-Ret phosphorylation, despite its undetectable binding to ^{125}I-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 ^{125}I-GDNF and ^{125}I-NTN (Fig. 4*A*). In all cases, the receptor chimeras showing the strongest binding to a given ligand were those retaining the central domain from the cognate receptor, *i.e.* X-1-X for GDNF and X-2-X for NTN. A similar pattern was observed in chimeras between GFRα1 and -4 analyzed by affinity labeling with ^{125}I-GDNF (Fig. 4*B*) and in chimeras between GFRα2 and -4 analyzed with ^{125}I-NTN and ^{125}I-PSP (data not shown). Together, these data demonstrate the importance of the central domain of GFRα receptors for ligand recognition. In contrast to the chimeras between GFRα1 and -3, the C-terminal domains of GFRα1, -2, and -4 could be exchanged without loss of binding (Fig. 4, *A* and *B*), probably because of their closer relative similarity (Table I). This indicates that the C-terminal domain can modulate ligand binding but is not an essential determinant of specificity.

The results of c-Ret phosphorylation assays in Neuro2A cells were again in general agreement with the binding data, *i.e.* the chimeras with the highest activity were the ones that retained the central domain from the cognate receptor (Fig. 4, *C* and *D*). 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. 4*C*). Interestingly, all chimeras between GFRα1 and GFRα2, as well as wild type GFRα1, supported some degree of c-Ret activation after NTN stimulation (Fig. 4*C*, *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 *Nar*I 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 ^{125}I-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. 5*A*). Conversely, the chimeras that retained binding to ^{125}I-NTN contained the first central subsegment from GFRα2 (Fig. 5*A*), 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 ^{125}I-GDNF and ^{125}I-NTN with comparable efficiency (Fig. 5*A*). 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. 5*B*), 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

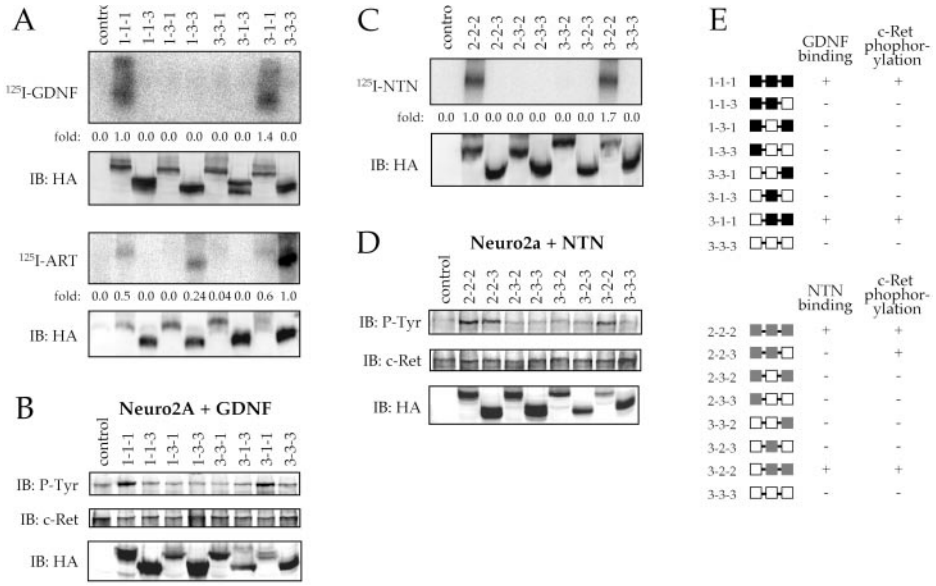

FIG. 3. **The N-terminal domain is not a determinant of ligand binding specificity.** *A*, affinity labeling of chimeras between GFRα1 and GFRα3 receptors with ^{125}I-GDNF and ^{125}I-ART as indicated. The *lower panels* show aliquots of cell lysates analyzed with HA antibodies by Western blotting. *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 ^{125}I-NTN. *D*, phosphorylation of c-Ret induced by NTN in Neuro2A cells expressing chimeras between GFRα2 and GFRα3 receptors. *E*, summary of results.

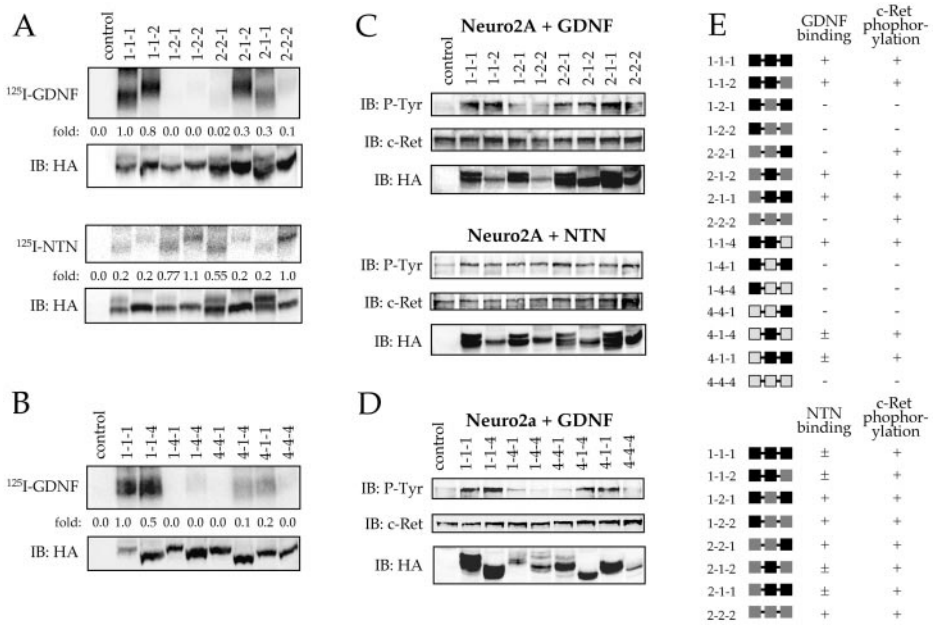

FIG. 4. **The central domain is a crucial determinant of ligand binding specificity.** *A*, affinity labeling of chimeras between GFRα1 and GFRα2 receptors with ^{125}I-GDNF and ^{125}I-NTN as indicated. The *lower panels* show aliquots of cell lysates analyzed with HA antibodies by Western blotting. *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 ^{125}I-GDNF. *C*, phosphorylation of c-Ret induced by GDNF and NTN in Neuro2A cells expressing chimeras between GFRα1 and GFRα2 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.

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. 6*A*). The ΔN161 deletion construct was however unable to bind GDNF (Fig. 6*B*), 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 ^{125}I-NTN at a level comparable with that of wild type GFRα2 (Fig. 6*C*). 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

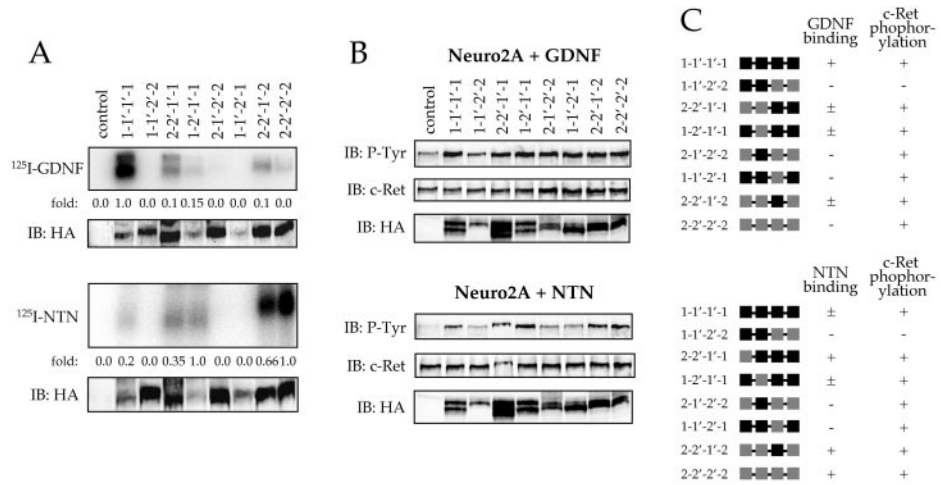

FIG. 5. **Binding determinants for GDNF and NTN reside in discrete subcentral domains of GFRα1 and GFRα2.** *A*, affinity labeling of chimeras between the subcentral domains of GFRα1 and GFRα2 receptors with ^{125}I-GDNF and ^{125}I-NTN as indicated. The *lower panels* show aliquots of cell lysates analyzed 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′-1 for GDNF and 2–2′-2′-2 for NTN) was set to one. *IB*, immunoblot. *B*, phosphorylation of c-Ret induced by GDNF and NTN in Neuro2A cells expressing chimeras between the subcentral domains of GFRα1 and GFRα2 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. *C*, summary of results.

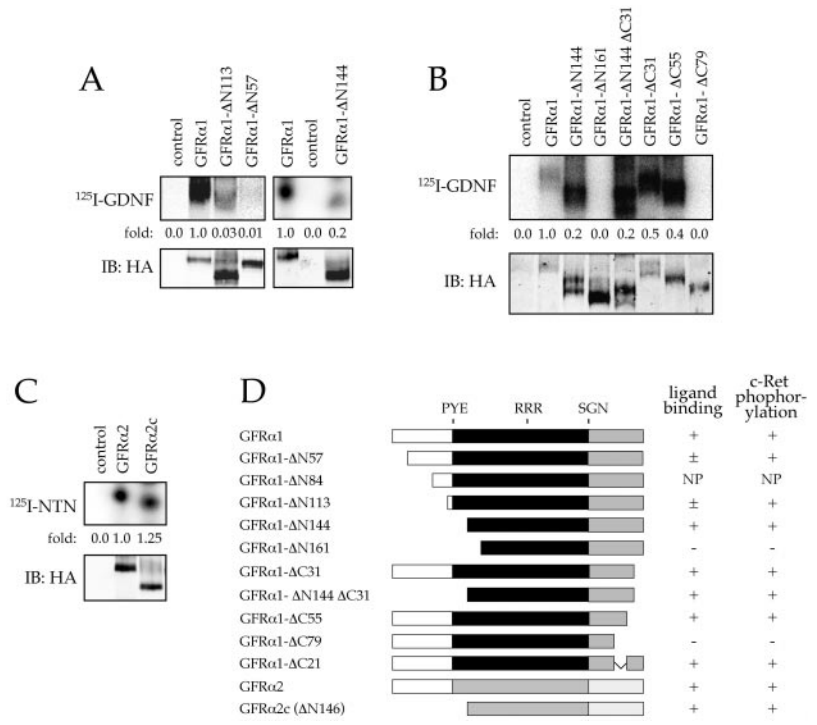

FIG. 6. **Truncated GFRα receptors lacking the N-terminal domain and part of the C-terminal domain retain ligand binding.** *A*, affinity labeling of N-terminal deletion constructs of GFRα1 with ^{125}I-GDNF. The *lower panels* show aliquots of cell lysates analyzed with HA antibodies by Western blotting. *Numbers below* the *lanes* indicate relative binding normalized to expression levels. Control (no GFRα receptor) was set to zero, whereas the interaction with wild type GFRα1 was set to one. *IB*, immunoblot. *B*, affinity labeling of N- and C-terminal deletion constructs of GFRα1 with ^{125}I-GDNF. *C*, affinity labeling of the GFRα2 N-terminal deletion construct ΔN146 (GFRα2c) with ^{125}I-NTN. *D*, linear diagrams of N- and C-terminal deletion constructs of GFRα1 and GFRα2. The central domain between PYE and SGN is *shaded*, and the C-terminal region is *hatched*. A qualitative summary of the results is shown to the *right*.

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 ΔC31 and ΔC55 retained their ability to bind ^{125}I-GDNF (Fig. 6*B*). A compound deletion mutant lacking 144 residues in the N terminus and 31 residues in the C terminus (GFRα1-ΔN144ΔC31) was also able to bind GDNF (Fig. 6*B*). 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. 6*B*). 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. 6*D*). 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 ΔN57, ΔN113, and ΔN144 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 ^{125}I-GDNF, the ΔC31 and ΔC55 C-terminal deletion mutants, as well as the ΔN144ΔC31 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

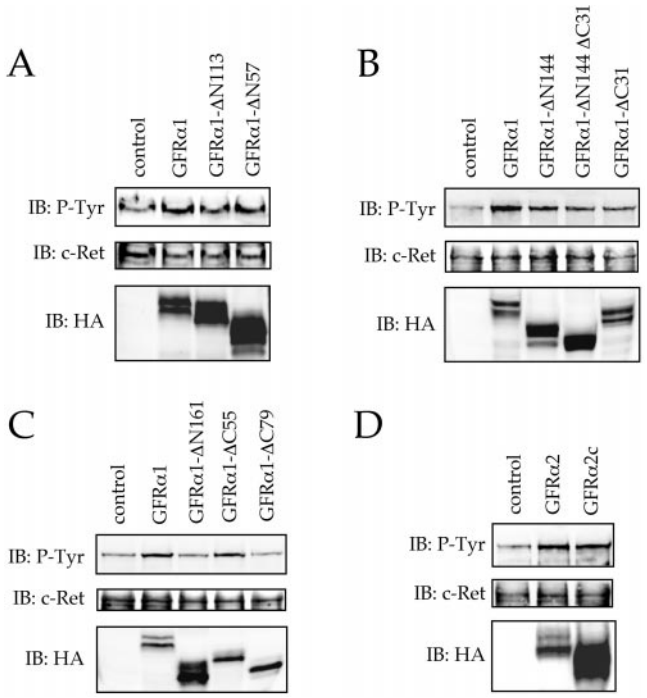

FIG. 7. **The N-terminal domain of GFRα receptors is dispensable for ligand-dependent activation of c-Ret.** *A–C*, tyrosine phosphorylation of c-Ret stimulated by GDNF in Neuro2A cells expressing N- and C-terminal deletion constructs of GFRα1. The *middle panel* shows reprobing of the same filter with anti-c-Ret antibodies. The *lower panel* shows aliquots of cell lysates analyzed with HA antibodies. *IB*, immunoblot. *D*, tyrosine phosphorylation of c-Ret stimulated by NTN in Neuro2A cells expressing the GFRα2 N-terminal deletion construct ΔN146 (GFRα2c).

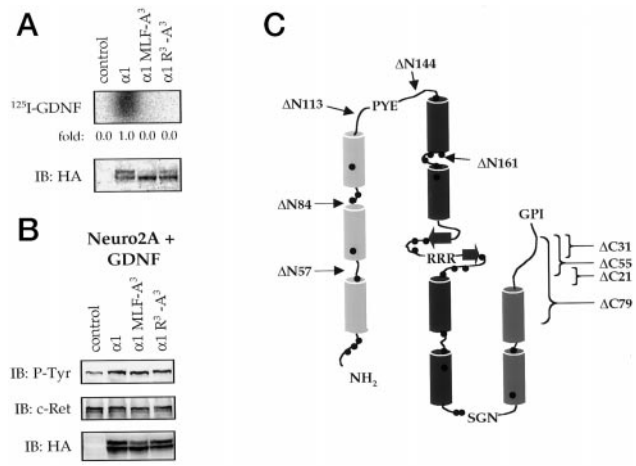

FIG. 8. **Distinct hydrophobic and positively charged residues in the central domain of GFRα1 mediate binding to GDNF.** *A*, affinity labeling of wild type GFRα1 (α1) and MLF and RRR mutants with ^{125}I-GDNF. The *lower panel* shows aliquots of cell lysates analyzed with HA antibodies by Western blotting and confirms equal levels of expression among the different constructs. *IB*, immunoblot. *B*, phosphorylation of c-Ret induced by GDNF in Neuro2A cells expressing MLF and RRR GFRα1 mutants. The *middle panel* shows reprobing of the corresponding filter with anti-c-Ret antibodies. The *lower panels* show aliquots of cell lysates analyzed with HA antibodies. *C*, summary scheme with salient features of the predicted secondary structure of GFRα receptors. *Solid black dots* denote cystine residues. Predicted α helices are represented as *cylinders*, and β strands as *flat arrows*. Amino acid sequences used for domain boundaries (PYE, RRR, and SGN) are indicated. The positions of N- and C-terminal deletions are indicated. Ligand binding specificity was found to reside in the central domain (*dark gray helices*), with contribution from immediately adjacent sequences in the C-terminal domain (*gray helices*). The N-terminal domain of GFRαs (*light gray helices*) does not play a major role in ligand binding or c-Ret interactions. Distinct residues involved in the binding of GFRα1 to GDNF include the RRR triplet in the center of the molecule and the MLF sequence in the first predicted β strand.

N-terminal residues, was also able to mediate NTN-dependent c-Ret activation (Fig. 7*D*). 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. 7*C*).

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-A^3) abolished binding of GFRα1 to GDNF (Fig. 8*A*). Moreover, replacement of RRR into an alanine triplet (α1 R^3-A^3) also impaired the ability of GFRα1 to interact with GDNF (Fig. 8*A*). 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-A^3 and R^3-A^3 GFRα1 mutants retained the capacity of mediating substantial levels of c-Ret phosphorylation in the presence of GDNF (Fig. 8*B*), 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

less conserved.

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. 8*C*). 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 GFRα1, -2, or -4 could not be substituted for that of GFRα3 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 GFRα1 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 GFRα1 by cyanogen bromide digestion and Edman microsequencing indicates that the central region that was most relevant to ligand binding in GFRα1 forms a core structural unit reinforced by disulfide bridges.[2] 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 GFRα4 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 GFRα2c splice variant and the mammalian GFRα4 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 GFRαs 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 GFRα1 with GDNF. The MLF and RRR triplets in the central region of GFRα1 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 GFRα1, suggesting that they could be interacting with the MLF and RRR triplets, respectively, that we identified here in GFRα1. 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 GFRα1 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, 20–22). Thus, either a residual affinity between suboptimal pairs of GDNF ligands and GFRαs is still capable of recruiting c-Ret to stabilize the complex, or, as previously proposed, GFRαs 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 GFRαs 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 GFRα1, *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 GFRα1 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.

Acknowledgments—We thank Bob Gordon, Stefan Massuré, and Miroslav Cik for providing PSP and ART ligands, Ella Cederlund and Thomas Bergman for help with HPLC and amino acid sequencing, Ann-Sophie Nilsson and Annika Ahlsén for technical assistance, and Xiaoli Li-Ellström for secretarial help.

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