GFRα-2 and GFRα-3 Are Two New Receptors for Ligands of the GDNF Family

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The receptor for glial cell line-derived neurotrophic factor (GDNF) consists of GFRα-1 and Ret. Neurturin is a
GDNF-related neurotrophin whose receptor is presently unknown. Here we report that neurturin can bind to
either GFRα-1 or GFRα-2, a novel receptor related to GFRα-1. Both GFRα-1 and GFRα-2 mediate neurturin-
induced Ret phosphorylation. GDNF can also bind to either GFRα-1 or GFRα-2, and activate Ret in the presence of either binding receptor. Although both ligands interact with both receptors, cells expressing GFRα-1 bind GDNF more efficiently than neurturin, while cells expressing GFRα-2 bind neurturin preferentially. Cross-linking and Ret activation data also suggest that while there is cross-talk, GFRα-1 is the primary receptor for GDNF and GFRα-2 exhibits a preference for neurturin.

We have also cloned a cDNA that apparently codes for a third member of the GFRα receptor family. This putative receptor, designated GFRα-3, is closely related in amino acid sequence and is nearly identical in the spacing of its cysteine residues to both GFRα-1 and GFRα-2. Analysis of the tissue distribution of GFRα-1, GFRα-2, GFRα-3, and Ret by Northern blot reveals overlapping but distinct patterns of expression. Consistent with a role in GDNF function, the GFRαs and Ret are expressed in many of the same tissues, suggesting that GFRαs mediate the action of GDNF family ligands in vivo.

Both neurturin and GDNF have been shown to promote the survival of sympathetic neurons derived from the superior cervical ganglia and of sensory neurons of both the nodose and dorsal root ganglia (1, 6, 9, 10, 13, 15, 16). Neurturin and GDNF mRNAs are widely distributed in a variety of both neuronal and non-neuronal tissues of embryos and adults (6, 15–18). Both are found in brain, kidney, and lung, whereas neurturin mRNA is also expressed at high levels in neonatal blood (15–21).

The striking structural and biological similarities between GDNF and neurturin suggest that their action may be mediated by the same or related receptors. The receptor for GDNF consists of a complex of GDNF Family Receptor α-1 (GFRα-1, previously abbreviated as GDNFR-α) and the Ret protein tyrosine kinase (PTK) (22, 23). GFRα-1 is a glycosyl phosphatidylinositol-anchored cell surface molecule. GFRα-1 binds to GDNF but cannot signal independently since it lacks a cytoplasmic domain. GDNF signaling is accomplished via association of the complex of GDNF and GFRα-1 with Ret, resulting in activation of the Ret kinase.

GFRα-1 mRNA is widely distributed in neuronal and non-neuronal tissues and is expressed throughout embryonic development to adulthood, implying a broad spectrum of biological functions (23, 24). The other component of the GDNF receptor complex, Ret, is a receptor type PTK encoded by the ret proto-oncogene (25). Ret mRNA and protein are highly expressed in the central and peripheral nervous systems, as well as in the kidney (26, 27). Various mutations in the ret gene are associated with human inherited diseases, including familial medullary thyroid carcinoma (28, 29), multiple endocrine neoplasia type 2A (MEN2A) and 2B (MEN2B) (28–32), and Hirschsprung’s disease (33, 34). Targeted disruption of the ret gene in knockout mice results in severe phenotypic defects, including renal agenesis or severe dysgenesis and lack of the entire enteric nervous system (35). These defects are very similar to those caused by GDNF null mutations (36–38), implying that GDNF-mediated signaling through Ret is required for the development of these tissues. Much less severe defects, however, were detected in a number of neuronal structures in which both GFRα-1 and Ret were expressed, such as the trigeminal and vestibular ganglia, the facial motor nucleus, the substantia nigra, and the locus coeruleus (35–38). This suggests that either GDNF signaling is not required for the embryonic development of these structures, or that some unknown signaling molecules similar to GDNF or Ret may exist that can substitute for them. Alternatively, the embryonic development of these tissues may rely completely on other as yet unknown signaling systems.

In this paper we report the cloning of GFRα-2 and GFRα-3, two novel receptors related to GFRα-1, and provide evidence
that GFRα-2 is a receptor for both GDNF and neurturin. Our data also indicate that GFRα-1 is a receptor for neurturin as well as for GDNF. We describe a related cDNA that codes for a protein, GFRα-3, that shares significant amino acid homology with both GFRα-1 and GFRα-2 and is likely to be a third member to the family of receptors for GDNF-related ligands.

EXPERIMENTAL PROCEDURES

Cloning of GFRα-2 and GFRα-3—A search of the GenBank data base for sequences related to GFRα-1 resulted in the identification of a single related EST, H12981.Gb_Est1. Primers corresponding to nucleotides 47–65 (5′-AAAGCGTCGGCTCGAGGTCGTTG-3′) and 244–265 (5′-CTCATAGGAGCA GCTTGGTTAGAAA-3′) of H12981.Gb_Est1 were synthesized and used for RT-PCR with human fetal brain mRNA (CLONTECH, catalog number 64019-1) as the template. A 218-nucleotide fragment was amplified, subcloned into pBlueScript (Stratagene, La Jolla, CA), and used for RT-PCR with human fetal brain mRNA (CLONTECH, catalog number NA931) in conjunction with chemiluminescence reagents (Amersham, Arlington Heights, IL) according to the manufacturer’s instructions. Rat and mouse DNA was labeled with 32P using a Random Primed DNA Labeling Kit (Boehringer Mannheim, Indianapolis, IN) according to the manufacturer’s instructions. Oligonucleotide primers were selected for expression of the plasmid by growth in 400 µg/ml G418 (Sigma). G418-resistant clones were expanded and assayed for GFRα-2 expression by Northern blot using the GFRα-2 cDNA as probe. Expression of GFRα-2 in individual clones was confirmed by binding to [125I]NTN.

Binding of [125I]NTN and [125I]GDNF to NNR-9 and NNR-38 cells were carried out as described previously (40). Briefly, cells were seeded 1 day before the assay in 24-well Costar tissue culture plates precoated with polyornithine at a density of 3 × 104 cells/cm2. Cells were placed on ice for 5–10 min, washed once with ice-cold buffer (Dubelco’s modified Eagle’s medium containing 25 mM HEPES, pH 7.0), and incubated at 4 °C in 0.2 ml of binding buffer (washing buffer containing 2 mg/ml bovine serum albumin) containing 50 µl [125I]NTN or [125I]GDNF in the presence or absence of 1000 nM unlabeled ligand for 4 h. Cells were washed 4 times with 0.5 ml of ice-cold washing buffer and lysed with 0.5 ml of 1 M NaOH. The lysates were counted in a 1470 Wizard Automatic Gamma Counter (Wallac Inc., Gaithersburg, MD).

Chemical Cross-linking—The coding regions of the first 455 amino acids of the human GFRα-1 and the first 451 residues of human GFRα-2 cDNAs were fused in-frame with a DNA fragment encoding the Fc region of human IgG2a, using the ligase system (Life Technologies Inc.) according to the manufacturer’s directions. Transfected cells were selected for expression of the plasmid by growth in 400 µg/ml G418 antibiotic (Sigma). G418-resistant clones were expanded and assayed for GFRα-2 expression by Northern blot using the GFRα-2 cDNA as probe. Expression of GFRα-2 in individual clones was confirmed by binding to [125I]NTN.

RESULTS

Cloning and Sequence Analysis of GFRα-2 and GFRα-3—A human expressed sequence tag (EST) with significant homology to GFRα-1 was found by a search of the publicly available...
nucleic acid sequence data bases. Oligonucleotides corresponding to the ends of this EST were synthesized and used in a reverse transcription-polymerase chain reaction (RT-PCR) with human fetal brain mRNA as the template. A fragment of the expected length was isolated, labeled, and used as a hybridization probe to screen a human fetal brain cDNA library. The longest clone isolated in this manner was sequenced and found to contain an open reading frame coding for a 464-amino acid protein related in sequence to GFRα-1. We have named this protein GDNF Family Receptor α-2 (GFRα-2). The oligonucleotides described above were also used to screen pools from a rat photoreceptor cDNA library by PCR and a product of the expected length was obtained from a single pool. An individual cDNA clone from this pool was identified by hybridization to the radiolabeled human GFRα-1 PCR product and sequenced. This clone contained an open reading frame coding for a 460-amino acid peptide that is nearly identical to human GFRα-2 and almost certainly represents its rat ortholog.

Publicly available sequence data bases were searched using GFRα-1 and GFRα-2 as query sequences and a short EST with homology to both GFRα-1 and GFRα-2 was found. Oligonucleotides corresponding to the ends of this EST were used as primers in RT-PCR with total rat embryo RNA as the template. A 225-nucleotide fragment was amplified, cloned into a plasmid vector, and sequenced to verify that it corresponded to the original GFRα-1/GFRα-2-related EST. Plasmid DNAs isolated

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**FIG. 1.** GFRα-1, GFRα-2, and GFRα-3 amino acid sequence comparison. The amino acid sequences of the human GFRα-1, GFRα-2, and GFRα-3 are aligned and a consensus sequence is shown above the three receptor sequences. **Upper case letters** in the consensus sequence indicate amino acids that are conserved in all three receptors, **lower case letters** indicate that two of the three receptors share that amino acid, and **dots** indicate all three receptors have a different amino acid at that position. Predicted signal peptide sequences and the hydrophobic COOH-terminal regions of all three receptors are underlined. Conserved cysteines are highlighted. Potential N-glycosylation sites are shown in **boldface** and are outlined by **boxes** if conserved between two receptors.
Fig. 2. Binding of neurturin and GDNF to NNR-9 and NGR-38 cells. NGR-38, NNR-9, and parental Neuro-2a cells were incubated with 50 pM of either $[^{125}I]$GDNF (A) or $[^{125}I]$NTN (B) in the absence (light gray bars) or presence (dark gray bars) of 500 nM unlabeled GDNF or neurturin at $4^\circ$C for 2 h. The unbound ligands were removed at the end of the incubation and the radioactivity associated with the cells determined. The number of femtomoles of cell-associated radiolabeled ligand is plotted for each experiment.

Fig. 3. Chemical cross-linking of neurturin and GDNF to GFRα-1 and GFRα-2 receptors. CM containing GFRα-1/Fc (GFRα-1) or GFRα-2/Fc (GFRα-2) fusion proteins were incubated with 2 nM $[^{125}I]$NTN (N) or $[^{125}I]$GDNF (G) in the presence (+ unlabeled) or absence (− unlabeled) of neurturin or GDNF. The bound receptor-ligand complexes were chemically cross-linked by the addition of 1 mM BS3, precipitated with Protein A-Sepharose and analyzed by SDS-PAGE as shown in the figure. The solid arrow indicates the ~110-kDa cross-linked species. The open arrow marks the ~220-kDa complexes. 

from pools of an E15 rat embryo cDNA library were screened by PCR with the same oligonucleotides and a single positive pool was found. Clones from this pool were screened by hybridization to the radiolabeled 225-nucleotide PCR fragment to isolate a single positive clone. Sequence analysis of the 1.8-kb insert revealed an open reading frame coding for a 397-amino acid peptide related to both GFRα-1 and GFRα-2. We have designated this putative receptor GFRα-3.

An alignment of the amino acid sequences of rat GFRα-1, GFRα-2, and GFRα-3 is shown in Fig. 1. The overall amino acid sequence identity among the three receptors is approximately 30–50%. GFRα-1 and GFRα-2 are somewhat more closely related to each other (48% identity) than they are to GFRα-3 (35% and 33% identity, respectively). Hydrophobic regions are found at both the amino and carboxyl termini of all three molecules (underlined, Fig. 1) and the amino-terminal hydrophobic regions have the characteristics expected for signal peptide sequences (43). The carboxyl-terminal hydrophobic region of GFRα-1 is known to be involved in glycosyl phosphatidylinositol linkage to the cell membrane (22, 23), and it is likely that the corresponding regions in GFRα-2 and GFRα-3 serve the same purpose. The most striking feature of the sequence alignment is the conservation of 28 cysteine residues among all three receptors (highlighted, Fig. 1), indicating that these proteins probably have similar three-dimensional structures. Several potential N-glycosylation sites are present in the GFRαs (shown in boldface, Fig. 1), but none are found at the same position in all three receptors. GFRα-1 and GFRα-2 share sites at positions 365 and 427 that are not found in GFRα-3, and GFRα-2 shares a possible site with GFRα-3 at positions 322–323 (Fig. 1).

Both Neurturin and GDNF Bind to NNR-9 and NGR-38 Cells—NRG-38 (22) and NNR-9 are cell lines derived from mouse Neuro-2a cells, which endogenously express high levels of Ret (44), but no detectable GFRα-1 (22) or GFRα-2 (data not shown). NGR-38 expresses high levels of recombinant GFRα-1 and binds GDNF specifically (22). High level expression of GFRα-2 in NNR-9 cells was confirmed by Northern blot (data not shown). NNR-9, NGR-38, and Neuro-2a cells were incubated with $^{125}$I-labeled NTN or GDNF in the absence or presence of excess unlabeled ligand. As shown in Fig. 2A (n = 5), more $^{125}$I[GDNF bound to NGR-38 cells (that express GFRα-1) than to NNR-9 cells (that express GFRα-2). The binding of $^{125}$I[GDNF to both NGR-38 and NNR-9 cells was effectively inhibited by an excess of either unlabeled NTN or GDNF. For $^{125}$I[NTN binding, the situation was reversed; more $^{125}$I[NTN bound to NNR-9 cells than to NGR-38 cells (Fig. 2B, n = 5). In addition, $^{125}$I[NTN binding was effectively inhibited by unlabeled neurturin but was much less affected by the addition of unlabeled GDNF (Fig. 2B). No significant binding of either $^{125}$I[NTN or $^{125}$I[GDNF to the parental Neuro-2a cells was detected (Fig. 2).

Cross-linking of Neurturin and GDNF to GFRα-1 and GFRα-2—Our binding experiments suggest that both neurturin and GDNF interact with GFRα-1 and GFRα-2. However, lack of an antibody specific for GFRα-2 has hampered our effort to further study these interactions. To overcome this difficulty, we generated plasmids that transiently express GFRα-1/Fc and GFRα-2/Fc fusion proteins when transfected into 293T cells. Conditioned medium (CM) containing either GFRα-1/Fc or
GFRα-2 and GFRα3, Two Novel Receptors for GDNF Family Ligands

GFRα-2/Fc fusion proteins was incubated with \([^{125}\text{I}]\)NTN or \([^{125}\text{I}]\)GDNF, chemically cross-linked, and then precipitated directly using Protein A-Sepharose beads. The immunoprecipitates were then analyzed by reducing SDS-PAGE (Fig. 3). When either \([^{125}\text{I}]\)GDNF or \([^{125}\text{I}]\)NTN was incubated with media containing GFRα-1/Fc, broad major bands centered at −110 and −220 kDa were observed (Fig. 3). Similar species were observed when either radiolabeled ligand was incubated with media containing GFRα-2/Fc (Fig. 3). The −110-kDa bands correspond to the expected molecular weights of monomeric GFRα-1/Fc or GFRα-2/Fc associated with either \([^{125}\text{I}]\)GDNF or \([^{125}\text{I}]\)NTN. The −220-kDa species probably represent a dimeric GFRα-1/Fc or GFRα-2/Fc complexed to either \([^{125}\text{I}]\)GDNF or \([^{125}\text{I}]\)NTN. When CM from mock transfected cells were used, no cross-linked band was precipitated by Protein A-Sepharose (data not shown). In all cases, addition of the corresponding unlabeled ligand to each sample resulted in a reduction in intensity of the major cross-linked bands (Fig. 3).

**Neurturin Induces Ret Autophosphorylation in Cells That Express GFRα-1—**The ability of neurturin to associate with GFRα-1 suggests that neurturin, like GDNF, might be able to activate Ret through GFRα-1. To examine this possibility, we tested the ability of neurturin to induce Ret autophosphorylation in the GFRα-1 expressing NGR-38 cells (22). NGR-38 cells were treated with various concentrations of neurturin up to 50 nM, lysed, and the lysates immunoprecipitated with anti-Ret antibody. A 170-kDa band, corresponding to phosphorylated Ret are indicated by an arrow.

**Expression of GFRα-1, GFRα-2, and GFRα-3 in Adult Rat—**The expression of GFRα-1, GFRα-2, and GFRα-3 mRNAs in adult rat tissues was examined by blot hybridization analysis. GFRα-1 mRNA is widely expressed, with high levels found in lung, brain, liver, kidney, and spleen (Fig. 6A). Expression is also detectable in heart and among the tissues examined is absent only in muscle and testis. Two distinct size transcripts are observed and their relative amounts vary among the tissues. The 3.6-kb transcript is predominant in liver, lung, heart, and spleen while comparable amounts of the 3.6- and 8.5-kb transcripts are present in brain and kidney. The tissue distribution of GFRα-2 mRNA is similar to that of GFRα-1 (Fig. 6B). GFRα-2 expression is highest in lung, spleen, and brain, with lesser amounts in kidney and heart. The most striking difference is the lack of GFRα-2 expression in liver. The size of the GFRα-2 transcripts is approximately 3.6 kb, similar to the smaller of the two GFRα-1 transcripts. The expression of GFRα-3 mRNA is highest in kidney and is notably absent in brain (Fig. 6C). Detectable expression of GFRα-3 is also present in spleen, lung, liver, and heart. The transcript size for GFRα-3 is somewhat smaller (~2.1 kb) than that observed for GFRα-1 and GFRα-2.

**Expression of GFRα-1, GFRα-2, and GFRα-3 in Mouse Embryo—**Developmental expression of GFRα-1, GFRα-2, and GFRα-3 mRNA was examined in mouse on embryonic days 7, 11, 15, and 17. Expression of the 3.6-kb transcript of GFRα-1 is first apparent at E11, seems to decrease somewhat at E15, but then increases dramatically by E17 (Fig. 7A). A minor amount...
of the 8.5-kb GFR-α-1 mRNA can be detected on E11, but no expression of this transcript is detected thereafter. The expression of the 3.6-kb GFR-α-2 transcript is barely detectable at E11, but increases gradually through E17 (Fig. 7B). Expression of the 2.1-kb GFR-α-3 mRNA is not detected at E7, but is quite strong by E11 (Fig. 7C). After E11, expression decreases and remains constant from E15–E17.

DISCUSSION

The classic mechanism of signal transduction for receptor PTKs involves a direct interaction of the PTK with its ligand that results in receptor dimerization and activation (45). A novel alternative to this process was revealed by the characterization of the receptor for GDNF (22, 23). GDNF induces signaling through the Ret PTK by first binding to GFR-α-1, a glycosyl phosphatidylinositol-linked cell surface molecule lacking a transmembrane domain. Only after binding to GFR-α-1 can GDNF interact with Ret and induce activation of the Ret kinase. Here we report the cloning of GFR-α-2, a receptor closely related to GFR-α-1. The 48% amino acid identity between GFR-α-1 and GFR-α-2, along with the nearly complete conservation of cysteine residues suggests that these two molecules have very similar three-dimensional structures and are likely to have similar biological functions.

We have demonstrated that both neuritin and GDNF can bind to either GFR-α-1 or GFR-α-2. Furthermore, binding of GDNF or neuritin to either receptor results in association of the ligand with Ret and consequent activation of the Ret PTK. Although both ligands bind both receptors, the evidence indicates that GDNF is the preferred ligand for GFR-α-1 and that neuritin is preferred by GFR-α-2. GFR-α-1-expressing NGR-38 cells are able to bind more GDNF than neuritin while the binding preference is reversed for GFR-α-2-expressing NNR-9 cells (Fig. 2). Consistent with these results, GDNF cross-links more effectively to GFR-α-1/Fc fusion proteins than to GFR-α-2/Fc fusions, while neuritin’s cross-linking preference is reversed (Fig. 3). The relative abilities of GDNF and neuritin to stimulate Ret phosphorylation in conjunction with cells expressing either GFR-α-1 or GFR-α-2 is consistent with both the binding and cross-linking data. In the GFR-α-1 expressing NGR-38 cells, the concentration of GDNF required to stimulate Ret autophosphorylation is approximately 100-fold lower than that observed for neuritin (Fig. 4). Conversely, in the GFR-α-2 expressing NNR-9 cells, neuritin stimulates Ret autophosphorylation at 100-fold lower concentrations than those required by GDNF (Fig. 5). Taken together, the binding, cross-linking, and phosphorylation data described above strongly suggest that although there is some cross-talk, GFR-α-1 is the primary receptor for GDNF while GFR-α-2 is the primary receptor for neuritin. We may also speculate that GFR-α-3 has an as yet undiscovered cognate ligand of its own.

It is interesting that the binding of [125I]GDNF to both GFR-α-1 and GFR-α-2 can be replaced by both unlabeled GDNF and neuritin, but that of [125I]NTN can only be inhibited by unlabeled neuritin (Fig. 2). A possible explanation for this result is the existence of two distinct sites: a neuritin-binding site that does not bind GDNF very effectively and a GDNF-binding site that can harbor either GDNF or neuritin. Human GDNF has a 33-amino acid highly basic motif at its NH2 terminus that is absent in human neuritin (16). This domain makes the GDNF molecule larger and more positively charged than neuritin and could prevent GDNF from competing for a smaller, less negatively charged neuritin-binding site. However, neuritin might still be able to compete for a larger, less restrictive GDNF site. If this model is correct, truncated forms of GDNF may be able to effectively compete with neuritin for its binding site.

We have presented evidence that both GFR-α-1 and GFR-α-2 utilize the Ret PTK to effect transmembrane signaling. The question of whether other signaling components, possibly Ret-like molecules, might interact with the GFRαs is still open. If all GDNF-like ligands signal exclusively through Ret, the same intracellular pathways should be triggered by all members of the family. While the tissue-specific distribution and the developmental expression of the GFRαs may determine where and when each ligand should be in action, the final biological readout should not vary. The near identity of the phenotypes resulting from the targeted disruption of the GDNF and Ret genes implies that if other signaling partners for GDNF exist, they are not important during development of these tissues. Further comparison of the biological activities of GDNF and neuritin should provide clues regarding the existence of alternate signaling partners for the GFRαs.

The properties of GFR-α-1 and GFR-α-2 suggest that they define a new family of ligand binding receptors (the GFRαs) for GDNF-like molecules. We have also described the isolation of a cDNA clone, GFR-α-3, that has sequence homology to both GFR-α-1 and GFR-α-2 and probably represents a third member...
of the GFRα family. It is not clear whether or not GFRα-3 as well as any other yet to be discovered members of the GFRα receptor family will be capable of binding GDNF and/or neur-turin and mediating phosphorylation of Ret. We can speculate, however, that additional members of the GDNF-like family of ligands exist that may bind the GFRαs. Cross-talk among receptors and ligands has been seen in other families of receptor PTKs, such as the Trk, epidermal growth factor, and fibroblast growth factor families. Single receptors have also been reported to be shared equally by two ligands, such as TrkB by BDNF and NT-4/5. Our data suggest that this is also a feature of the GDNF ligand family and the GFRα receptor family of molecules. Such cross-talk among receptors and ligands, however, makes it very difficult to determine the physiological role of each ligand or receptor. Further elucidation of the patterns of expression of the GFRα family receptors will help us to advance our knowledge in this field. Targeted disruption of the genes for the GFRαs and GDNF-like molecules will provide more valuable insights into the biological roles of this receptor ligand family.

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