Functional Mapping of Receptor Specificity Domains of Glial Cell Line-derived Neurotrophic Factor (GDNF) Family Ligands and Production of GFRα1 RET-specific Agonists*

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The glial cell line-derived neurotrophic factor (GDNF) family ligands (GFLs) (GDNF, neurturin, artemin, and persephin) are critical regulators of neurodevelopment and support the survival of midbrain dopaminergic and spinal motor neurons in vitro and in animal disease models making them attractive therapeutic candidates for treatment of neurodegenerative diseases. The GFLs signal through a multicomponent receptor complex comprised of a high affinity binding component (GDNF family receptor α-component (GFRα1-GFRα4)) and the receptor tyrosine kinase RET. To begin characterization of GFR receptor specificity at the molecular level, we performed comprehensive homologue-scanning mutagenesis of GDNF, the prototypical member of the GFLs. Replacing short segments of GDNF with the homologous segments from persephin (PSPN) (which cannot bind or activate GFRα1-RET or GFRα2-RET) identified sites along the second finger of GDNF critical for activating the GFRα1-RET and GFRα2-RET receptor complexes. Furthermore, introduction of these regions from GDNF, neurturin, or artemin into PSPN demonstrated that they are sufficient for activating GFRα1-RET, but additional determinants are required for interaction with the other GFRs. This difference in the molecular basis of GFL-GFRα specificity allowed the production of GFRα1-RET-specific agonists and provides a foundation for understanding of GFL-GFRαRET signaling at the molecular level.

The glial cell line-derived neurotrophic factor (GDNF) family ligands (GFLs) are distant members of the TGF-β superfamily that are potent neurotrophic factors in vitro and are critical for the development of distinct neuronal populations in vivo (1). There are currently four known GFLs, GDNF (2), neurturin (NRTN (3)), artemin (ARTN (4)), and persephin (PSPN (5)). Each GFL is a demonstrated survival factor for dopaminergic ventral midbrain neurons cultured from the embryo (2, 4–6). Furthermore, GDNF, NRTN, and PSPN have also been shown to support the survival of motor neurons in culture (5, 7–9). Therefore there is considerable interest in the therapeutic potential of these molecules for use in treating neurodegenerative diseases such as Parkinson’s disease and amyotrophic lateral sclerosis, which is underscored by the success of GDNF in relieving disease symptoms in several animal models of Parkinson’s disease (10). However the difficulty of delivering these large polypeptides to their intended site of action in the central nervous system makes it desirable to understand the molecular basis of their action in attempts to produce minimal agonists of the GFL receptor system.

The GFLs signal through a unique multicomponent receptor complex, consisting of a high affinity glycosylphosphatidylinositol-anchored binding component (GFRα1-GFRα4) and the receptor tyrosine kinase RET. Extensive in vitro and in vivo experimentation has supported the hypothesis that for each GFL there is a preferred GFRα receptor, to which the GFL binds with highest affinity and most potently activates RET. These preferred interactions are GDNF-GFRα1, NRTN-GFRα2, and ARTN-GFRα3 (4, 9, 11, 12). PSPN does not bind or activate any of the mammalian GFRαs, but does bind to a protein currently only identified in chicken called GFRα4 (5, 13, 14). However, despite these preferential interactions, there is also clear cross-talk between the different ligand-receptor pairs. The known alternative interactions are NRTN-GFRα1, ARTN-GFRα1, and GDNF-GFRα2 (4, 11, 15, 16). As GFRα1 and GFRα2 are often expressed by the same or closely neighboring neurons (6, 17), it is still unclear through which receptor exogenously administered GDNF and NRTN mediate their neurotrophic actions on various neuronal populations. Whereas the in vitro interactions between the different GFLs and GFRαs are now relatively well understood, the molecular basis of this specificity and cross-talk is unknown (18). Furthermore, the precise details of receptor complex formation and stoichiometry are also poorly characterized. The crystal structure of GDNF indicates that it is a disulfide-bonded dimer that is significantly similar to the structure of TGF-β2, as predicted by the cysteine spacing of its primary sequence (19–21). However, the structure itself yields only speculative information regarding receptor binding surfaces. Furthermore, analogy to other TGF-β superfamily members regarding receptor binding surfaces would likely be unfounded as the receptors used by GDNF and the TGF-βs are drastically different and likely hold no structural similarity.

To characterize the structural requirements of GDNF for interacting with its receptors, we performed homologue-scanning mutagenesis, replacing short blocks (~8 amino acids) of GDNF sequence with the homologous blocks from PSPN, which cannot bind or activate GFRα1-RET or GFRα2-RET. This screen identified two critical regions for the GDNF-GFRα1...
Mapping of GDNF Family Ligand-Receptor Interaction Domains

interaction and at least one additional region critical for the alternate GDNF-GFRα2 interaction. These blocks are discontinuous along the primary sequence of GDNF but are directly adjacent along the second finger of GDNF when mapped to the crystal structure. We further demonstrate that these two regions from any of the GFRα1 agonists (GDNF, NRTN, or ARTN) are sufficient to activate GFRα1-RET in the context of the PSPN molecule and that these chimeric mutants function as GFRα1-RET-specific agonists in vitro. Finally, we identify an additional region that is critical for the NRTN-GFRα2 and ARTN-GFRβ3 interactions. These data identify a putative receptor interaction site shared by the GFLs to activate GFRα1-RET and identify additional determinants required for GDNF and NRTN to activate GFRα2-RET and for ARTN to activate GFRα3-RET.

EXPERIMENTAL PROCEDURES

Mutagenesis, Synthesis, and Purification of GFL Mutants—All mutants were produced by fusion polymerase chain reaction mutagenesis. The amino acid sequence of rat GDNF, mouse NRTN and PSPN, and human ARTN was used for all constructs. Polymerase chain reaction products were directly cloned into the MvuI and XhoI sites of plasmid pCB6 (22), and the inserts were sequenced entirely. For mutant G-hf-GDNF the 6His-FLAG tag was inserted between the third and fourth residue of mature GDNF, making the sequence (N-... RLRK SPD-HHHHHHDDYKDDDD-KQAL-...). For mutant N-hf-GDNF, residues 1–38 were truncated from mature human and attached to the prepro-region of NRTN. The resulting sequence was (N-... RRAR PQA-HH-HHHDDYKDDDD-RCG...). For N-hf-ARTN, residues 1–13 were truncated from mature human ARTN and attached to the prepro-region of NRTN, with the resulting sequence (N-... RRAR PQA-HH-HHHDDYKDDDD-RGCR...). For mutant P-hf-PSPN, the tag was inserted between the third and fourth residue of mature PSPN, resulting in N-... RLPR ALA-HHHHHHDDYKDDDD-GSRC...-...C. Expression plasmids for homologue-scanning mutants were produced as above by fusion polymerase chain reaction, and all were based on the N-hf-GDNF construct.

For mutant protein production in COS cells, expression plasmids were transfected using the DEAE-dextran/Chloroquine method (23). COS cells were plated onto 10- or 15-cm dishes, transfected, and switched to Dulbecco’s modified Eagle’s medium containing 1% fetal calf serum. After 4–6 days, conditioned medium was collected and either concentrated using Centriprep-10 concentrators (Amicon) or purified by nickel chromatography (Qiagen). Proteins were visualized by immunoblotting with anti-FLAG M2 monoclonal antibody (Sigma). Relative quantities of FLAG-tagged proteins were determined by an enzyme-linked immunosorbent assay also using the anti-FLAG M2 antibody. Briefly, purified proteins or conditioned medium were blotted to Nunc-Immunosorb microtiter plates overnight at 4 °C. Plates were washed (3 x in Tris-buffered saline, 0.03% Tween 20), blocked (blocking solution: Tris-buffered saline, 1% bovine serum albumin) for 1 h at 25 °C, washed again (5 x), and then incubated with a 1:500 dilution of anti-FLAG M2 antibody in blocking solution for 1.5 h at 25 °C. After another wash (5 x), plates were incubated with horseradish peroxidase-conjugated anti-mouse antibody (Amicon) and visualized using the ECL Plus kit (Amersham). Densities of bands were quantitated using the ImageQuant program (Molecular Dynamics). The “C/D” dimer from 1AGQ is shown, with the missing segment of the 2 interaction. These blocks are discontinuous along the primary sequence of GDNF but are directly adjacent along the second finger of GDNF when mapped to the crystal structure. We further demonstrate that these two regions from any of the GFRα1 agonists (GDNF, NRTN, or ARTN) are sufficient to activate GFRα1-RET in the context of the PSPN molecule and that these chimeric mutants function as GFRα1-RET-specific agonists in vitro. Finally, we identify an additional region that is critical for the NRTN-GFRα2 and ARTN-GFRβ3 interactions. These data identify a putative receptor interaction site shared by the GFLs to activate GFRα1-RET and identify additional determinants required for GDNF and NRTN to activate GFRα2-RET and for ARTN to activate GFRα3-RET.

RESULTS

The N-terminal Extension of GDNF Is Not Required for Activity—An alignment of the mature rat GDNF with mouse NRTN, ARTN, and PSPN is shown in Fig. 1. Rat GDNF was the basis for all mutagenesis in this study because its structure has been determined (21). The largest notable difference between the GFLs is the N-terminal extension before the first structural cysteine, which varies from 40 amino acids in GDNF to only 5 amino acids in NRTN and PSPN, and shows little similarity between the different family members. Furthermore GDNF, NRTN, and ARTN have multiple RXR consensus subtilisin-like protease convertase cleavage sites (27), and therefore multiple isoforms of these extensions are possible. The ones shown are consistent with N-terminal sequencing of GDNF and NRTN (2, 3), the single cleavage site in PSPN (5), and therapy with the cotransfected lacZ reporter. For the receptor complex formation assay, Neuro2a cells were transiently transfected using Superfect with an expression plasmid for rat GFRα1 with a FLAG tag inserted by polymerase chain reaction mutagenesis after the signal sequence. Cells were treated with 25 ng/mL of the indicated factor for 6 h; the cells were washed once with cold phosphate-buffered saline, lysed, and immunoprecipitated using an anti-FLAG M2 monoclonal antibody conjugated to agarose (Sigma). After washing, the immunoprecipitated samples were visualized by immunoblotting with an anti-RET antibody (C-9, Santa Cruz Biotechnology). The NBL-S proliferation assay and the SH-SYSY differentiation assay were performed as described (4).
Mapping of GDNF Family Ligand-Receptor Interaction Domains

Fig. 1. Primary sequence alignment of the mature GFLs. A, rat GDNF is shown with mouse NRTN, ARTN, and PSPN. Secondary structure elements ("α" for α-helix, "β" for β-strand, and 3 for 310-helix) as determined for GDNF (21) are indicated above the alignment. The regions delineated for homologue-scanning mutagenesis are in colored blocks, with the name of the region ("F1a-b" for finger 1, "Ha-d" for heel, and "F2a-d" for finger 2) below the given block. The color of each block corresponds to the color scheme shown in B, B, representation of backbone of the GDNF dimer showing the first (blue) and second (red) fingers and the heel region (green) of the molecule.

and a cleavage site conserved between mouse and human ARTN (4).

To determine if the large N-terminal extension of GDNF is required for its activity, we took advantage of the differential processing of GDNF and NRTN by mammalian cells. Chimeric constructs with the pre-pro-region from NRTN attached to GDNF were generated, and we assessed both their processing and their ability to activate the GFRα1-RET receptor complex (Fig. 2). Tandem 6X histidine and FLAG tags were inserted after the RXXR cleavage site of GDNF or NRTN to allow purification and tracking of the proteins. When expressed in COS cells, similar quantities of tagged GDNF with its own pro-region (G-hf-GDNF) or the NRTN pro-region (N-hf-GDNF) could be purified from conditioned medium using nickel chromatography (Fig. 2B). Only species corresponding to the expected processing events were observed in the medium, indicating that the pro-domains of NRTN and GDNF are sufficient to direct proper processing of their different N-terminal extensions.

To assess the ability of these proteins to activate the high affinity GDNF receptor, GFRα1-RET, we utilized the Gal4-Elk1/Gal4-luciferase reporter system as described previously (4). This system monitors the level of RET activation of the mitogen-activated protein kinase pathway, which induces the Elk1/Gal4-luciferase reporter, giving a facile measure of receptor activation (28, 29). The reporter plasmids, together with the receptor activation assay described above. Ten GDNF mutants (termed "GPG" mutants) were generated by replacing blocks of nonconserved sequence from PSPN into GDNF (Fig. 3, see Fig. 1 for sequence changes). This method allows comprehensive mutagenesis of sites responsible for the difference in the ability of GDNF and PSPN to activate GFRα1-RET, and is highly likely to maintain structural integrity of the mutants because the replacements are from a homologous (and likely structurally similar) protein. All mutants were produced at similar quantities when transfected into COS cells, and the processing of each appeared normal (Fig. 3B).

Comparison of the GPG mutants with GDNF revealed that although many mutants showed slightly decreased activity, only one (GPG-F2a) completely lost the ability to activate the GFRα1-RET receptor (Fig. 4A). This region maps to the bottom of the GDNF molecule along the second finger (Fig. 4B) (21).

The reporter plasmids with the indicated GDNF construct, were cotransfected with the receptor activation assay described above. Ten GDNF mutants (termed "GPG" mutants) were generated by replacing blocks of nonconserved sequence from PSPN into GDNF (Fig. 3, see Fig. 1 for sequence changes). This method allows comprehensive mutagenesis of sites responsible for the difference in the ability of GDNF and PSPN to activate GFRα1-RET and is highly likely to maintain structural integrity of the mutants because the replacements are from a homologous (and likely structurally similar) protein. All mutants were produced at similar quantities when transfected into COS cells, and the processing of each appeared normal (Fig. 3B).

Comparison of the GPG mutants with GDNF revealed that although many mutants showed slightly decreased activity, only one (GPG-F2a) completely lost the ability to activate the GFRα1-RET receptor (Fig. 4A). This region maps to the bottom of the GDNF molecule along the second finger (Fig. 4B) (21). The only other notable mutant that consistently showed activity 60% or lower than full-length GDNF was GPG-F2c, which
maps to the strand adjacent to the region F2a along the bottom of the molecule.

It is well established that GDNF is also capable of binding and activating the GFRα1-RET receptor, albeit with a lower affinity than NRTN (11, 15, 16), whereas PSPN cannot (5, 13). Therefore, to compare the molecular determinants of GDNF required for GFRα1 versus GFRα2 interaction, we tested the ability of this same panel of mutants to activate the GFRα2-RET receptor complex. Several of the GPG mutants were significantly more attenuated in their ability to activate GFRα2 versus GFRα1 (Fig. 1A). As before, mutant GPG-F2c was unable to activate the receptor. Interestingly, mutant GPG-F2c, which was the second most affected mutant in activating GFRα1-RET, showed essentially no activation of GFRα2-RET. Finally, the mutant that showed the most contrast in its ability to activate GFRα1-RET versus GFRα2-RET was mutant GPG-Ha, which was severely affected only in its ability to activate GFRα2-RET. Interestingly, mapping these three regions to the GDNF crystal structure reveals that they form a continuous surface, with residues from F2a and F2c from one monomer directly adjacent to region Ha from the heel of the other monomer, suggesting a possible receptor-ligand interaction surface (Fig. 4D). In summary, the above mutagenesis data suggest that two adjacent regions of GDNF located along the second finger are necessary for interaction with both GFRα1 and GFRα2 (regions F2a and F2c) and that additional regions appear to be required for GDNF interaction with GFRα2.

Identification of Regions F2a and F2c from GDNF, NRTN, or ARTN as Sufficient for Activating the GFRα1-RET Receptor Complex—The homologue-scanning mutagenesis data suggest that two adjacent regions of GDNF are critical for activating the GFRα1-RET receptor. However, using only loss of function analysis it is difficult to assess whether critical receptor-contact residues are being altered directly or if the mutations induce structural changes that alter receptor contact surfaces elsewhere on the molecule. To identify if any of the critical regions identified above (either alone or in combination) are sufficient to allow binding and activation of the GFRα1-RET receptor complex, we generated PSPN mutants with the putative critical regions from GDNF replacing the corresponding regions of PSPN. We then determined if these “PGP” mutants had gained the ability to activate the GFRα1-RET receptor complex (Fig. 5).

We focused initially on two candidate regions, F2a and F2c...
Mapping of GDNF Family Ligand-Receptor Interaction Domains

along the second finger of GDNF, because mutant GPG-F2a was entirely inactive, and mutant GPG-F2c was the only other mutant that consistently showed decreased activation of GFRα1-RET. Replacement of either region F2a or F2c alone from GDNF into PSPN was not sufficient to allow the resulting mutants (PGP-F2a or PGP-F2c) to activate GFRα1-RET. However, when both regions were placed into PSPN the resulting mutant (PGP-F2ac) gained the ability to activate the GFRα1-RET receptor at a level comparable to full-length GDNF (Fig. 5B). Furthermore, treatment of GFRα1-transfected Neuro2a cells (which endogenously express Ret) with either GDNF or mutant PGP-F2ac induced comparable GFRα1-RET complex formation, demonstrating that PGP-F2ac had gained the ability to bind and induce receptor complex formation (Fig. 5C). To determine if mutant PGP-F2ac was also able to elicit biological responses comparable to GDNF, we tested its ability to induce proliferation in the NBL-S neuroblastoma cell line, which expresses GFRα1 and RET and responds to GFRα1-RET agonists (GDNF, NRTN, ARTN) by proliferation (Fig. 5D) (4). Mutant PGP-F2ac purified from transiently transfected COS cell-conditioned medium induced robust proliferation of NBL-S cells, similar to GDNF at all doses, whereas PSPN did not. Furthermore, PGP-F2ac was also able to induce neurite outgrowth in the GFRα1-RET-expressing neuroblastoma line SH-SY5Y similar to GDNF (Fig. 5E) (4).

NRTN and ARTN are also able to activate GFRα1-RET (4, 30), and therefore we examined if the same regions (F2a and F2c) from these two additional GFRα1 agonists are also sufficient to activate the GFRα1-RET receptor (Fig. 6). Interestingly, the corresponding PSPN mutants with regions F2a and F2c from NRTN and ARTN were also capable of activating the GFRα1-RET receptor complex, at levels comparable to GDNF (Fig. 6C). This indicates that elements of regions F2a and F2c from all the known GFRα1 agonists are sufficient to activate GFRα1-RET when placed in the context of PSPN. Furthermore, these regions appear to be sufficient only for GFRα1-RET activation, as the same mutants did not activate GFRα2-RET comparably to GDNF (Fig. 6C). Mutants PNP-F2ac and PGP-F2ac did elicit low level activation of GFRα2-RET, whereas PAP-F2ac did not. This is consistent with the ability of GDNF and NRTN, but not ARTN, to activate GFRα2-RET (4) and indicates that regions F2a and F2c are also
involved in binding and activating GFRα2-RET but that additional regions are required for full activation.

**Additional Determinants Are Critical for NRTN to Activate GFRα2-RET and for ARTN to Activate GFRα3-RET**—The observation that mutants PGP-F2ac and PNP-F2ac cannot fully activate GFRα2-RET is consistent with the homologue-scanning mutagenesis above (Fig. 4), which suggested that regions in addition to F2a and F2c are required for GDNF to activate GFRα2-RET. In particular, mutant GPG-Ha was significantly attenuated in its ability to activate GFRα2-RET (Fig. 4), whereas it showed a minor loss in the ability to activate GFRα1-RET (Fig. 4; 27%).

Therefore, consistent with the homologue-scanning mutagenesis above, these data indicate that regions F2a and F2c of GDNF and NRTN are only sufficient to activate GFRα1-RET, and additional regions are required for full activation of GFRα2-RET (region Ha and perhaps others).

**ARTN is a recently identified member of the GFLs that can activate GFRα1-RET, and the only member of the family that can activate GFRα3-RET** (4). As shown above, regions F2a and F2c from ARTN are sufficient to activate GFRα1-RET. In an initial attempt to characterize the molecular determinants of the ARTN-GFRα3 interaction, we examined the ability of PSPN-ARTN chimeras to activate the GFRα3-RET receptor complex (Fig. 7, C and D). As expected, N-hf-ARTN gave robust activation of GFRα3-RET, whereas N-hf-GDNF showed no activity. Mutant PAP-F2ac, which is capable of activating GFRα2-RET (see Fig. 6), was also unable to activate...
GFRα3-RET, indicating that like the NRTN-GFRα2 interaction, the ARTN-GFRα3 interaction requires regions in addition to F2a and F2c. To determine if region Ha was also involved in the ARTN-GFRα3 interaction, we generated a PSPN mutant with regions Ha, F2a, and F2c from ARTN (Fig. 7C). Interestingly, whereas mutant PAP-F2ac was entirely inactive, mutant PAP-Ha/F2ac regained significant activity (usually 70–80% of full length ARTN, Fig. 7D). Therefore, similar to the NRTN-GFRα2 interaction, the ARTN-GFRα3 interaction requires molecular determinants from both the heel and finger 2 regions of the molecule, whereas interaction of GDNF, NRTN, or ARTN with GFRα1 only requires regions F2a and F2c from finger 2.

PSPN/GDNF Chimeras Function as GFRα1-specific Agonists in Vitro—The homologue-scanning mutagenesis and gain of function experiments above demonstrate that for the GFRα1-RET agonists (GDNF, NRTN, and ARTN), regions F2a and F2c are sufficient for interaction with GFRα1 but that additional regions are required for interaction with GFRα2 and GFRα3 (Figs. 6 and 7). This suggests that the PSPN mutants containing only regions F2a and F2c from GDNF, NRTN, or ARTN may function as GFRα1-RET-specific agonists. To test this idea, we developed a cell survival assay using rat cerebellar granule cells in culture. These cells do not express endogenous RET or GFRα coreceptors and can be transfected efficiently, therefore providing a model system of cell survival with defined receptor components. Cerebellar granule cells transfected with RET or GFRα1 alone do not survive in the absence of high potassium or in the presence of GDNF; however, cells cotransfected with GFRα1 and RET survive in the presence of GDNF at levels comparable to the control condition of high potassium plus serum (Fig. 8A) (24). Consistent with other assays, cerebellar granule cells transfected with GFRα1-RET or GFRα2-RET survive in the presence of either GDNF or NRTN, but not PSPN (Fig. 8B). However, mutant PGP-F2ac was only capable of supporting the survival of GFRα1-RET-transfected, but not GFRα2-RET-transfected cells. This is consistent with its minimal ability to activate the GFRα2-RET receptor in fibroblasts (Fig. 6C) and indicates that unlike GDNF and NRTN, mutant PGP-F2ac functions as a GFRα1-RET-specific agonist in this in vitro cell survival paradigm.

**DISCUSSION**

We report a comprehensive mutagenesis analysis of GDNF, the prototypical member of the GDNF family of neurotrophic
factors, in an attempt to characterize at the molecular level the interaction between GDNF and its receptors GFRa1 and GFRz2. This analysis revealed similar regions are sufficient for GFRz1 interaction by all known GFRa1 ligands; GDNF, NRTN, and ARTN. Furthermore, we found that additional regions are required for activating GFRz2-RET and GFRz3-RET, which allowed the production of GFRz1-RET-specific agonists as tested in receptor activation and cell survival assays.

The N-terminal Extension of GDNF Is Not Required for Activity—The N-terminal extension before the first structural cystine-knot cysteine is highly variable among the TGF-β superfamily members. In the case of TGF-β2, it exists as a short α-helix and is stabilized by an additional disulfide bond relative to other members of the superfamily (19, 20). However, in GDNF and OP-1, the only other members of the TGF-β superfamily that have been crystallized, the 35–37 residue N-terminal extension was unresolved and therefore does not adopt a consistent conformation in solution (21, 31). We found that truncating the N-terminal extension did not affect the ability of GDNF to activate its receptors. This is consistent with experiments characterizing monoclonal antibodies against GDNF that also suggested the N-terminal extension is not required for receptor binding or bioactivity (32). The role of the N-terminal extension of GDNF remains unclear; however, because it does not appear to be involved in receptor interaction, possibilities such as its importance for stability and/or binding additional proteins or to extracellular matrix elements warrant consideration.

Characteristics of the Putative GFRz Interaction Site of GDNF—The mutagenesis reported here indicates that critical residues for GFRz receptor interaction and specificity are located in the second finger region of the GDNF molecule. However, whereas the use of homologue-scanning mutagenesis is ideally suited for identifying sites involved in differential receptor specificity, it cannot delineate all residues involved in receptor binding, as some of these may be identical in GDNF and PSPN. The fact that residues from regions F2a and F2c are sufficient when placed in the context of PSPN to activate GFRz1-RET indicates that residues in these regions are likely to be directly involved in binding to GFRz. Although it is possible that the GFLs also contact RET in the active receptor complex, the fact that all members of the GFLs, including PSPN, appear to signal through RET makes it doubtful that the regions identified by our mutagenesis scheme are involved in RET interaction directly (4, 14). Because we focused on functional activation of the GFRz-RET receptor complex rather than receptor binding assays, we cannot exclude the possibility that some of the nonfunctional mutants produced here may still be capable of binding to the GFRz coreceptors. Such mutants could potentially function as receptor antagonists, and we are currently investigating this possibility.

Current evidence suggests that the stoichiometry of the active receptor complex for GDNF is (GDNF)1(GFRz)2(RET)3 (33). Residues from regions F2a and F2c project essentially from the bottom the GDNF structure and form symmetric sites on both monomers of the molecule (see Fig. 4B). Interestingly, residues from region Ha (which is critical for GDNF-GFRz2 and ARTN-GFRz3 interaction) lie directly adjacent to regions F2a and F2c from the finger 2 region of the opposing monomer and form continuous surfaces along the side and bottom of the molecule (see Fig. 4C). Because these regions are all critical for GFRz specificity, we suggest that the molecular surface formed by the heel of one monomer and the second finger of the adjacent monomer form GFRz binding surfaces on GDNF.

Comparison of structure-function studies from other TGF-β and cystine-knot superfamily members reveals common themes in the location of receptor binding surfaces. The best characterized of these is the binding of vascular endothelial growth factor with its receptors KDR and Flt-1, where extensive mutagenesis and co-crystallization have been performed (34, 35). Mutagenesis of vascular endothelial growth factor identified a cluster of residues critical for KDR binding, several of which were along the adjacent β-strands of the second finger, similar to regions F2a and F2c identified here for GDNF. Furthermore, even though the orientation of the monomers in the vascular endothelial growth factor dimer is different from GDNF, the receptor binding site on vascular endothelial growth factor involves residues from finger 2 and the analogous heel region of the opposing monomer (34). Finally, mutagenesis of TGF-β indicates that the binding affinity of the different TGF-β isoforms for the TGF-β type II receptor is also determined by residues along the second finger of the molecule, analogous in location to region F2c (36, 37). Therefore it is possible that although the receptor systems and dimerization modes are strikingly different for these different cystine-knot superfamily proteins, the location of their receptor interaction surfaces may be quite similar.

Differences in GFRz1-GFRz2-GFRz3 Interaction—In our analysis of the structural determinants of GDNF required for activating its two functional receptors, GFRz1-RET and GFRz2-RET, we observed that only regions F2a and F2c were required for activating GFRz1-RET but additional regions (including region Ha) were required for activating GFRz2-RET. GFRz1-RET is the most promiscuous of the GFL receptors and is able to interact with three of the four known ligands. This is

![Fig. 8. Mutant PGP-F2ac functions as a GFRz1-RET-specific agonist in a neuronal survival assay. A, cerebellar granule cells dissected from 5-day-old rats were transfected with the indicated receptor components and cultured for 3 days in the presence of high potassium plus serum (K25-S), low potassium (K5), or low potassium plus GDNF (GDNF). Only neurons transfected with GFRz1 and RET together survived in the presence of GDNF. B, cerebellar granule cells transfected with GFRz1-RET or GFRz2-RET were cultured in the presence of the indicated factors. NRTN was bacterially produced and added at 50 ng/mL. All others were produced in COS cells and added as concentrated conditioned medium. Whereas GDNF and NRTN support the survival of neurons expressing GFRz1-RET or GFRz2-RET similarly, mutant PGP-F2ac only supports the survival of GFRz1-RET expressing neurons.](http://www.jbc.org/content/3319/5507/3419/F8.large.jpg)
consistent with GFRα1-RET having the most minimal requirements for being activated (regions F2a and F2c) and additional regions being required for activating GFRα2-RET and GFRα3-RET. It is of note that regions F2a and F2c from GDNF, NRTN, or ARTN conferred similar activity to PSPN in the receptor activation assay. It will be interesting to determine if these mutants confer similar GFRα1-binding characteristics to their parent constructs (PGP-F2ac with higher affinity than PNP-F2ac or PAP-F2ac) or if they all bind GFRα1 with similar affinity, suggesting that regions of NRTN and ARTN outside F2a and F2c may actually reduce their affinity for GFRα1.

Although region Ha is critical for the NRTN-GFRα2 and ARTN-GFRα3 interactions, PSPN mutants containing regions Ha, F2a, and F2c were not fully active, indicating that both additional regions are necessary or the binding determinants from NRTN and ARTN are not presented properly in the context of the PSPN molecule. A better understanding of receptor complex formation and stoichiometry and direct co-crystallization will be required to confirm and refine our understanding of the receptor binding surfaces of these molecules.

Utility of GFL Chimeras as GFRα1-specific Agonists—The GFLs are promising candidates as therapeutic agents for the treatment of neurodegenerative diseases, including Parkinson's disease. GDNF, NRTN, and ARTN all support the survival of cultured dopaminergic neurons of the embryonic ventral midbrain (2, 4, 6), and GDNF and NRTN have both shown to be effective in supporting the survival of midbrain dopaminergic neurons in animal injury models (6, 10). Several lines of evidence argue that the effects of GDNF, NRTN, and ARTN on dopaminergic ventral midbrain neurons are mediated through the GFRα1-RET receptor system. First, because GFRα3 is not expressed in the ventral midbrain and ARTN cannot utilize GFRα2, survival promotion of these neurons by ARTN is likely through its ability to activate GFRα1-RET (4). Second, GFRα2 expression is diffuse and weak in the para compacta region of the substantia nigra and does not colocalize with tyrosine hydroxylase staining neurons, in contrast to the significantly stronger expression of GFRα1, which does colocalize with tyrosine hydroxylase staining neurons (6). Finally, the ability of both GDNF and NRTN to support the survival of dopaminergic ventral midbrain neurons is lost in GFRα1 knockout mice, indicating that at least in the embryo the survival promotion of dopaminergic ventral midbrain neurons is only through GFRα1-RET signaling (38). Because there are several central and peripheral sites of GFRα2-RET expression that could lead to side effects in the treatment of patients with GDNF or NRTN, the GFL chimeras that function as GFRα1-RET-specific agonists described here (PGP-F2ac and particularly PAP-F2ac) could be therapeutically useful as they would minimize potential GFRα2-related side effects, while maintaining the ability to support survival and growth of the desired GFRα1-RET expressing target neuron populations.

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