Identification of the Key Amino Acids of Glial Cell Line-derived Neurotrophic Factor Family Receptor α1 Involved in Its Biological Function*

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Glia1 cell line-derived neurotrophic factor (GDNF) plays a critical role in neurodevelopment and survival of midbrain dopaminergic and spinal motor neurons in vitro and in vivo. The biological actions of GDNF are mediated by a two-receptor complex consisting of a glycosylphosphatidylinositol-linked cell surface molecule, the GDNF family receptor α1 (GFRα1), and receptor protein tyrosine kinase Ret. Although structural analysis of GDNF has been extensively examined, less is known about the structural basis of GFRα1 function. In this study, based on evolutionary trace method and relative solvent accessibility prediction of residues, a set of trace residues that are solvent-accessible was selected for site-directed mutagenesis. A series of GFRα1 mutations was made, and PC12 cell lines stably expressing different GFRα1 mutants were generated. According to the survival and differentiation responses of these stable PC12 cells upon GDNF stimulation and the GDNF-GFRα1-Ret interaction assay, residues 152NN153, Arg259, and 318NS118 in the GFRα1 central region were found to be critical for GFRα1 binding to GDNF and eliciting downstream signal transduction. The single mutation R259A in the GFRα1 molecule simultaneously lost its binding ability to GDNF and Ret. However, I152A/N153A or S316A/N317A/S318A mutation in the GFRα1 molecule still retained the ability to bind with Ret. These findings suggest that distinct structural elements in GFRα1 may be involved in binding to GDNF and Ret.

Most biological processes are governed by specific protein-protein interactions. Unfortunately, some macromolecular complexes do not yield x-ray quality co-crystals, and these complexes are frequently beyond the current limits of multidimensional NMR spectroscopy. Thus, structural knowledge is often limited to either receptor or ligand alone, which by itself lacks explicit binding site information. Even when the structure of a complex is available, it is very difficult to deduce the relative contribution of each individual residue to the total binding energy. In fact, the binding site is a distinct subset of the contact sites. Mutagenesis studies have shown that less than half of the residues buried in the binding interface may contribute ~90% of the total binding energy (1). Similarly, in the case of the neurotrophin system, three basic residues provide the critical binding determinants for interaction with their p75 receptor (2, 3). Mutational analysis, therefore, remains a mainstay of molecular determination of binding interfaces between receptor-ligand complexes.

The glial cell line-derived neurotrophic factor family ligands include GDNF1 (4), neurturin (5), artemin (6), and persephin (7). They are critical regulators of neurodevelopment (4) and support the survival of midbrain dopaminergic (6, 8) and spinal motor neurons (9, 10) in vitro and in animal disease models, making them attractive therapeutic candidates for treatment of neurodegenerative diseases (11, 12). GDNF is also an inducer and branching factor of ureteric buds during kidney development (13) and plays an important role in sperm cell development (14). The neurotrophic and morphogenic activities of GDNF are mediated by its interaction with a multicomponent receptor complex formed by the Ret receptor tyrosine kinase (15–18) and a glycosylphosphatidylinositol (GPI)-anchored accessory receptor, GDNF family receptor α1 (GFRα1) (19, 20). Four different GFRα receptors have been identified (GFRα1–4), and each of them prefers binding to one of the glial cell line-derived neurotrophic factor family ligands (21). GDNF binds GFRα1 with preferred high affinity. The unprocessed precursor of rat GFRα1 contains 468 amino acids, with a signal peptide at the amino terminus and a stretch of 38 hydrophobic amino acids at the carboxyl terminus. The hydrophobic cluster at the carboxyl terminus is preceded by a group of three small amino acids (Ala, Ser, and Ser), defining a cleavage/binding site for GPI linkage, suggesting that GFRα1 is an extracellular protein that is attached to the outer cell membrane (20). GDNF, GFRα1, and Ret bind together and subsequently lead to Ret dimerization and activation of the Ret tyrosine kinase. The

The abbreviations used are: GDNF, glial cell line-derived neurotrophic factor; GFRα1, GDNF family receptor α1; GPI, glycosylphosphatidylinositol; ET, evolutionary trace; PAFAH, platelet-activating factor acetylhydrolase; PBS, phosphate-buffered saline; RT-PCR, reverse transcription-PCR; GdmCl, guanidinium chloride.

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original model of GDNF signaling proposes a stringent division of labor between GFRα1 and Ret receptors, in which the latter transduces intracellular signaling but cannot bind ligand on its own, and the former binds ligand but is thought not to transduce signals in the absence of Ret. However, GDNF mutants deficient in GFRα1 binding are still able to activate Ret normally, suggesting that at least some Ret molecules are weakly associated with GFRα1 before GDNF binding (22). The precise details of receptor complex formation and stoichiometry of components within the complex remain unclear. The GDNF-GFRα1-Ret complex provides an attractive system in which to investigate protein-protein interactions involved in the assembly of multisubunit receptor complexes.

The x-ray crystal structure of GDNF (23) and its distinct structural elements involved in GFRα1 binding (12, 22, 24) have been determined. However, less is known about the structure and function analysis of GFRα1. One study has reported that the central region of GFRα receptors constitutes a novel binding domain for cysteine-knot superfamily ligands. The carboxyl-terminal segments adjacent to the central domain are necessary and have modulatory function in ligand binding (25). In our work, a set of residues in the central and carboxyl-terminal domain of GFRα1 were investigated for their involvement in functional receptor complex formation and signal transduction by alanine-scanning mutagenesis. The selected residues are evolutionarily conserved residues identified by the evolutionary trace method (26) and also have relatively high solvent accessibility in PHD prediction (27). After generating stable PC12 cell lines containing this series of GFRα1 mutants, we examined GDNF binding, Ret activation, and differentiation and survival responses to GDNF. Our results indicate that several amino acids in the GFRα1 receptor are critical for binding with GDNF and Ret and mediating the neurotrophic function of GDNF.

**EXPERIMENTAL PROCEDURES**

**Reagents**—The restriction endonucleases and T4 DNA ligase were purchased from MBI. TdT was obtained from Takara Biotechnology Co., Ltd. Mini-MTM DNA extraction system and gel extraction miniprep kit were from VIOGENE. Plasmid pBluescript SK(+) was obtained from Takara Biotechnology Co., Ltd. Mini-MTM DNA extraction system and gel extraction miniprep kit were from VIOGENE. Plasmid pBluescript SK(+), pCDNA3-GFRα1, and pCDNA3-Ret were generated previously in our laboratory. The poly-L-lysine, fetal calf serum, horse serum, and Dulbecco’s modified Eagle’s medium were purchased from Invitrogen. PC12 cells were the generous gift from the Cell Bank of Shanghai Institute of Cell Biology, Chinese Academy of Sciences. FuGENE 6.0 was purchased from Roche Applied Science. Anti-phosphotyrosine monoclonal antibody pY99, anti-GDNF mouse monoclonal antibody B-8, anti-GFRα1 rabbit polyclonal antibody H-70, and anti-Ret goat polyclonal C-20 antibodies were from Santa Cruz Biotechnology. Fluorescein isothiocyanate-conjugated chicken anti-rabbit IgG and rhodamine-conjugated donkey anti-goat IgG were from Chemicon International, Inc. GDNFs were prepared as described previously (28). All other chemicals were purchased from BBI.

**Evolutionary Trace Analysis, Secondary Structure Prediction, and Site-directed Mutagenesis**—Based on the principle of molecular evolution, sequence and structure information can be integrated by evolutionary analysis to determine functional sites in the protein. This method is now called evolutionary trace (ET) (26). The improved ET method (29, 30) was used to identify the functional epitopes in the members of the GFRα family. A total of 37 GFRα primary sequences were compiled following a BLAST search with the sequence of rat GFRα1 over the NCBI non-redundant data base. Multiple sequence alignment and dendrogram construction were then carried out by the ClustalX program (31). Secondary structure and residues relative to solvent accessibility predictions of rat GFRα1 were deduced using the PHD predict program (27).

The rat full-length GFRα1 cDNA was subcloned into pCDNA3.0. Single-stranded DNA from this plasmid was used as a template for oligonucleotide-based site-directed mutagenesis as described previously (32). All mutations were confirmed by DNA sequence analysis.

**Generation of Stable PC12 Cells Containing Mutant GFRα1 Receptor**—PC12 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 5% fetal bovine serum, 5% horse serum, and 0.05% glucose. All cells were grown at 37 °C in 5% CO₂. 1.5 × 10⁵ PC12 cells were plated in each well of a 12-well plate (Corning Glass) that had been coated with poly-L-lysine prior to transfection. After overnight incubation, the monolayer cell density would achieve 1.3 × 10⁵ cells were plated in each well of a 12-well plate pre-coated with poly-L-lysine. After attachment, the cells were exposed to 100 ng/ml GDNF. Seven days later, cell different effects of GDNF on cell differentiation were determined. PC12 cells possessing one or more neurites of a length more than twice the diameter of the cell body were scored as positive. Each value was the mean ± S.E. sampled from three independent experiments. For the survival assay, PC12 cells were seeded as described above. After attachment, the cells were switched to serum-free medium plus 100 ng/ml GDNF. Cell survival was quantified by staining with 10 μg/ml fluorescein diacetate (Sigma) after 48 h as described previously (33). Viable cells were determined by fluorescence microscopy. The value of the viable cell number in culture with the medium-containing serum represented our corrected 100% initial survival. Results are expressed as the percentage of cell counts with respect to the 100% initial value and show the mean ± S.E. of the percentages from three independent experiments.

**Reverse Transcription-PCR Analysis**—Cells were lysed in TRIzol, and the RNA concentration was measured photometrically. First strand cDNA was synthesized from 1 μg total RNA using (dT)₅ primer. The PCR primer pair 5′-ccacagcctgaggctgtg-3′ and 5′-tggggatcctttcgttcctactact-3′ corresponding to the specific part of GFRα1 was amplified to a 453-bp product. After an initial denaturing step of 5 min at 95 °C, the amplification was carried out for 30 cycles at 95 °C for 30 s, 57 °C for 30 s, and 72 °C for 30 s. A final amplification for 10 min at 72 °C finished the PCR. The PCR product was cloned into the pBSK vector and verified by DNA sequence analysis.

**Flow Cytometry**—Cells grown on coverslips were washed briefly with PBS and fixed with 4% paraformaldehyde for 20 min. Cells were blocked with 10% normal goat serum at room temperature for 20 min and then incubated with primary antibodies at 4 °C overnight followed by fluorescent secondary antibodies at room temperature for 45 min. Coverslips were mounted with 90% glycerol in PBS and examined by fluorescent microscopy.

**Site-directed Mutagenesis of Selected Residues in GFRα1**—The primary sequences of GFRα family members were retrieved by BLAST search and used for the evolutionary trace analysis. The dendrogram of these sequences separated them...
into four main clusters that each correlated with a different subgroup in this family from GFRα1 to e4 (data not shown). The globally invariant residues that are conserved in all the GFRα family members were identified; these residues are likely to contribute to important structural domains or some shared functions for the whole family. The class-specific residues of GFRα1, which are conserved in GFRα1 subgroup but are variable in other GFRα subgroups, are considered to be important for delineating the functional specificities of GFRα1 subgroup. The GFRα family receptors have a unique pattern of cysteine residues and lack many of the domains most commonly present in other receptors. Thus, this receptor family likely represents a structurally novel receptor class. Secondary structure and relative solvent accessibility predictions of residues of rat GFRα1 were done by PHD prediction. Our secondary structure predictions results were similar to those reported previously (25). According to the domain boundaries defined by those authors, we also divided the GFRα1 molecule into amino-terminal (three predicted helices), central (four predicted helices and two β-strands), and carboxyl-terminal (two predicted helices) domains of roughly 100, 200, and 100 residues, respectively (Fig. 1). The residues targeted for site-directed mutagenesis are trace residues, both globally invariant residues and class-specific residues, in the central domain or carboxyl-terminal segments adjacent to the central domain of GFRα1 with relatively high solvent accessibility. The selected residues that fit the criteria for mutagenesis are shown in Fig. 1. These residues may be on the protein surface and specify the functional epitopes in GFRα1 that interact with other molecules. Among them, Asn<sup>152</sup>, Arg<sup>171</sup>, Arg<sup>197</sup>, Arg<sup>259</sup>, and Ser<sup>318</sup> are globally invariant residues in the whole family, and Asn<sup>216</sup>, Lys<sup>206</sup>, Gln<sup>247</sup>, Asp<sup>248</sup>, Ser<sup>249</sup>, Glu<sup>270</sup>, Asp<sup>294</sup>, Ser<sup>316</sup>, Asn<sup>317</sup>, Leu<sup>326</sup>, Lys<sup>327</sup>, Asp<sup>334</sup>, and Lys<sup>339</sup> are class-specific residues of the GFRα1 subgroup. These residues were mutated into alanine, either individually or in combinations of two or three residues (Table I). Alanine is best suited to the scanning approach because it can accommodate most elements of the secondary structure of proteins and results in minimal structural distortion.

### The Effects of GDNF on PC12 Cells Stably Expressing GFRα1 and Ret—By stable selection, a series of PC12 cell lines stably expressing GFRα1 mutants and Ret were generated. In order to investigate the key amino acids of GFRα1 involved in its biological function, the effects of GDNF on cell differentiation and survival were evaluated by treating these PC12 cells with 100 ng/ml GDNF. Seven days after treatment, compared with PC12-Ret cell line (negative group) and PC12-GFRα1-Ret cell line (positive group), GDNF could significantly induce neurite outgrowth of PC12-GFRα1(R171A)-Ret, PC12-GFRα1-(R197A)-Ret, PC12-GFRα1(K206A)-Ret, PC12-GFRα1(E270A)-Ret, PC12-GFRα1(D284A)-Ret, PC12-GFRα1(L326A/K327A)-Ret, PC12-GFRα1(D334A)-Ret, and PC12-GFRα1(K339A)-Ret stable cells (p < 0.05 compared with the PC12-Ret group, Fig. 2, A and D). PC12-GFRα1(Q247A/D248A/S249A)-Ret stable cells gave an intermediate response (p < 0.05 compared with both PC12-Ret and PC12-GFRα1-Ret stable cells (Fig. 2, C and D)). However, GDNF could not induce neurite outgrowth in PC12-GFRα1(N152A/N153A)-Ret, PC12-GFRα1(R259A)-Ret, and PC12-GFRα1(S16A/N317A/S318A)-Ret stable cells (Fig. 2, B and D). The survival-promoting effects of GDNF on PC12-stable cells were consistent with differentiation responses of PC12-stable cells to GDNF (Fig. 3). Two days after treatment, GDNF could significantly promote survival of PC12-GFRα1-Ret, PC12-GFRα1(R171A)-Ret, PC12-GFRα1(R197A)-Ret, PC12-GFRα1(K206A)-Ret, PC12-GFRα1(E270A)-Ret, PC12-GFRα1(D284A)-Ret, PC12-GFRα1(L326A/K327A)-Ret, PC12-GFRα1(D334A)-Ret, and PC12-GFRα1(K339A)-Ret stable cells in serum-free medium. The survival-promoting effect of GDNF on PC12-GFRα1(Q247A/D248A/S249A)-Ret stable cells was intermediate (p < 0.05 compared with both PC12-Ret and PC12-GFRα1-Ret stable cells, Fig. 3). However, GDNF could not promote survival of PC12-GFRα1(N152A/N153A)-Ret, PC12-GFRα1(R259A)-Ret, and PC12-GFRα1(S16A/N317A/S318A)-

### Table 1

| GFRα1 variant | GDNF binding | Ret binding | Ret phosphorylation | Activation of differentiation | Promotion of survival |
|---------------|--------------|-------------|---------------------|-------------------------------|----------------------|
| Wild-type     | ++           | ++          | ++                  | ++                            | ++                   |
| N152A/N153A  | ++           | ++          | ++                  | ++                            | ++                   |
| R171A         | ++           | ++          | ++                  | ++                            | ++                   |
| R197A         | ++           | ++          | ++                  | ++                            | ++                   |
| K206A         | ++           | ++          | +                   | +                             | +                    |
| Q247A/D248A/S249A | ++ | ++ | + | + | + |
| K339A         | ++           | ++          | +                   | +                             | +                    |
| E270A         | ++           | ++          | ++                  | ++                            | ++                   |
| D284A         | ++           | ++          | ++                  | ++                            | ++                   |
| S316A/N317A/S318A | -- | -- | -- | -- | -- |
| L252A/K327A  | ++           | ++          | ++                  | ++                            | ++                   |
| D334A         | ++           | ++          | ++                  | ++                            | ++                   |
| K339A         | ++           | ++          | ++                  | ++                            | ++                   |
GFR growth in PC12 cells transfected with GDNF could not induce any neurite outgrowth in GFR112(K206A), GFR1 together with Ret. In addition, Western blot analysis confirmed that all GFR1 expression in these stable cell lines. The Western blots confirmed that all GFR1 mutants were equivalent (Fig. 5). Ret was also expressed in all stably transfected PC12 cell lines (Fig. 6).

After stimulation of GDNF for 7 days, PC12 cells stably expressing GFR1-Ret and possessing one or more neurites of more than twice the diameter of the cell body were scored as positive. Each value was the mean ± S.E. from three independent experiments. A, GDNF could induce neurite outgrowth in PC12 cells transfected with wild type GFR1, GFR1(R171A), GFR1(R197A), GFR1(K206A), GFR1(E270A), GFR1(D284A), GFR1(L326A/K327A), GFR1(D334A), and GFR1(K339A) together with Ret. B, GDNF could not induce any neurite outgrowth in PC12 cells transfected with GFR1(N152A/N153A), GFR1(R259A), and GFR1(S316A/N317A/S318A) together with Ret. C, GDNF only induced medium neurite outgrowth in PC12 cells transfected with GFR1(Q247A/D248A/S249A) together with Ret. D, the statistics of neurite outgrowth effects of GDNF on GFR1-Ret-PC12 cells. Each value is the mean ± S.E. sampled from three independent experiments. *, p < 0.05 compared with Ret-PC12 cells after GDNF stimulation. #, p < 0.05 compared with GFR1(WT)-Ret-PC12 cells after GDNF stimulation.

Ret stable cells in serum-free medium (Fig. 3).

Determination of Expression of GFR1 or GFR1 Mutants and Ret in PC12 Cells—To eliminate the possibility that the absence of response of PC12-GFR1(N152A/N153A)-Ret, PC12-GFR1(R259A)-Ret, and PC12-GFR1(S316A/N317A/S318A)-Ret stable cells to GDNF may be due to lack of expression of these GFR1 mutants, RT-PCR and Western blot analyses were performed to confirm appropriate expression in these PC12 cell lines. The transcription of GFR1 mutants was ascertained by RT-PCR, utilizing GFR1-specific primers that produced an expected amplified product of ~450 bp. The results showed GFR1 mutants mRNA were all transcribed in these PC12 stable cell lines (Fig. 4). In addition, Western blot analysis of PC12 cell lysates was performed using anti-GFR1 and anti-Ret antibodies to confirm GFR1 and Ret protein expression in these stable cell lines. The Western blots confirmed that all GFR1 mutants were expressed in the stable PC12 cell lines, and the expression levels between GFR1 and the various mutants were equivalent (Fig. 5). Ret was also expressed in all stably transfected PC12 cell lines (Fig. 6).

Immunofluorescence Cell Staining—It is well known that GFR1 protein is linked to the plasma membrane by a glycosylphosphatidylinositol anchor. Immunofluorescence microscopy was used to investigate whether GFR1 mutants localized to the cell surface. The results showed that GFR1 staining was bright and sharp along the cell surface of the stably transfected PC12 cells, with no staining in the control group (Fig. 7). These results suggested that the GFR1 mutant proteins expressed in transfected PC12 cells were properly localized on the cell surface.

Phosphorylation and GDNF-GFR1-Ret Interaction—To determine which residues of GFR1 are involved in binding to GDNF or Ret and to influence the activation of Ret tyrosine kinase, critical residues in the GFR1 receptor were investigated (Fig. 8).
kinase, a series of stable PC12 cells were treated with GDNF for 15 min, followed by immunoprecipitation with anti-GFRα1 or anti-Ret antibodies. Immunoprecipitates were analyzed by immunoblotting with anti-GDNF, anti-Ret, and antiphosphotyrosine antibodies, respectively. The Western blots were shown in Figs. 8–10, 10 and summarized in Table I. Combined mutants GFRα1(N152A/N153A) and GFRα1(S316A/N317A/S318A) lost their ability to bind to GDNF, and the other mutants displayed almost normal levels of GDNF binding ability compared with wild type GFRα1.

**Fig. 5.** Analysis of GFRα1 and its mutants protein expression in PC12 cells. Lysates from stable GFRα1-Ret-PC12 cells containing GFRα1 mutants were analyzed by Western blot with anti-GFRα1 antibodies.

**Fig. 6.** Analysis of Ret protein expression in GFRα1-Ret-PC12 cells. A series of GFRα1-Ret-PC12 cell lysates was analyzed by Western blot with anti-Ret antibodies.

A series of stable PC12 cells were treated with GDNF for 15 min, followed by immunoprecipitation with anti-GFRα1 or anti-Ret antibodies. The localization of GFRα1 mutant proteins expressed in PC12 cells were stained with anti-GFRα1 antibodies followed by fluorescein isothiocyanate-conjugated anti-rabbit IgG. A, wild type PC12 cells and PC12 cells transfected with Ret only. B, PC12 cells transfected with wild type GFRα1 or GFRα1 mutants together with Ret.

**Fig. 7.** Immunofluorescence cell staining of GFRα1-Ret-PC12 cells. The localization of GFRα1 mutant proteins expressed in PC12 cells were stained with anti-GFRα1 antibodies followed by fluorescein isothiocyanate-conjugated anti-rabbit IgG. A, wild type PC12 cells and PC12 cells transfected with Ret only. B, PC12 cells transfected with wild type GFRα1 or GFRα1 mutants together with Ret.

**Fig. 8.** GDNF binding capacity of GFRα1 mutants analyzed by immunoprecipitation/Western blotting. The lower panels show aliquots of cell lysates immunoprecipitated by anti-GFRα1 antibodies. The upper panels show immunoprecipitation of anti-GFRα1 antibodies immunoblotted by anti-GDNF antibodies. Mutants GFRα1(N152A/N153A), GFRα1(R259A), and GFRα1(S316A/N317A/S318A) lost their ability to bind to GDNF, and the other mutants displayed almost normal levels of GDNF binding ability compared with wild type GFRα1. **IB,** immunoblot.

**Fig. 9.** GDNF binding capacity of GFRα1 mutants analyzed by immunoprecipitation/Western blotting. The lower panels show aliquots of cell lysates immunoprecipitated by anti-GFRα1 antibodies. The upper panels show immunoprecipitation of anti-GFRα1 antibodies immunoblotted by anti-GDNF antibodies. Mutants GFRα1(N152A/N153A), GFRα1(R259A), and GFRα1(S316A/N317A/S318A) lost their ability to bind to GDNF, and the other mutants displayed almost normal levels of GDNF binding ability compared with wild type GFRα1. **IB,** immunoblot.
GDNF binding and are indispensable for ligand-stimulated Ret phosphorylation. However, they are not involved in binding to Ret. The residue Arg259 is essential in GDNF and Ret binding and Ret phosphorylation. Residues 247QDS249 appear not to be critical for binding to either GDNF or Ret but may influence the stability of the GDNF-GFRα1-Ret complex and then influence ligand-stimulated Ret phosphorylation. The rest of the residues tested (Arg171, Arg197, Lys206, Glu270, Asp284, 326LK327, Asp334, and Lys339) appear not to be critical for GFRα1 binding to GDNF and Ret.

The Effects of the Mutations on the Conformation and Stability of the Protein—In order to verify that the loss of activities of GFRα1 mutants is due to changes in binding and not simply due to unfolding of the proteins, CD experiments and unfolding tests under different concentrations of GdmCl of these proteins were performed to assess the effects of the mutations on the structure and stability of the protein. The far-UV CD spectra of the four mutants (GFRα1(N152A/N153A), GFRα1(Q247A/D248A/S249A), and GFRα1(S316A/N317A/S318A) are similar to that of wild type GFRα1 (Fig. 11A), indicating that the secondary structures remain unchanged upon mutations. The CD-monitored unfolding curves of the four mutants also show no significant changes (Fig. 11B), indicating that the four mutations do not affect the unfolding properties of GFRα1.

Discussion

In this study, alanine substitution was applied to the trace residues of the GFRα1 receptor with relatively high solvent accessibility to investigate their role in GDNF and Ret binding and subsequent receptor complex activation. The original evolutionary trace analysis is an all-or-none consensus sequence-based method that treats all columns with variable amino acid residues as non-conserved, regardless of the physicochemical similarity between them and thus may affect the sensitivity of this method (26). In this study, the improved ET method (29, 30) was used to identify the functional epitopes in GFRα1 family members. Amino acid exchange matrices were used to better tolerate variations in each column, and each sequence was weighted according to its level of similarity with others to prevent the over-representation of similar sequences in the protein data base.

It has been reported that the central domain of GFRα1 is a crucial determinant of ligand binding specificity and is critical for GDNF-induced neurotrophic function. The carboxyl-terminal segments adjacent to the central domain are necessary and have modulatory functions in ligand binding (25, 36). However, which residues in central domain and carboxyl-terminal segments are responsible for its biological function were not known. Based on the evolutionary trace method, secondary structure, and relative solvent accessibility predictions of residues, 12 sites in rat GFRα1 were selected for alanine mutagenesis. PC12 cells, which express low levels of endogenous Ret...
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GFRα1 for GDNF and Ret binding, respectively. We speculate that GFRα1 might pre-associate weakly with Ret in the absence of ligand, and GDNF stimulation enhances the GDNF-GFRα1-Ret complex formation. In our study, the GFRα1(N152A/N153A) mutation retained Ret binding capacity, which was consistent with the conclusion of a previous study (25) that the first 161 residues from the amino terminus of GFRα1 might be deleted without affecting the ability of GFRα1 to interact with Ret (25). These findings suggest that GFRα1 does have an interface directly interacting with Ret.

Arg259 in GFRα1 is crucial not only for GFRα1 and Ret binding but also for activation of the receptor complex. This residue is a globally invariant residue that is completely conserved in the entire GFRα family, suggesting a similar role for this site in all GFRα members. That a single mutation R259A in GFRα1 lost its ability to bind both GDNF and Ret suggests that a point mutation in one subunit of heterotrimeric complex could lead to simultaneous loss of binding with the other two subunits. A comparable example for this situation is Lis1. A point mutation of H149R or S169P in Lis1 led to loss of binding to either PAFAH1B2 or PAFAH1B3 subunit and impairment in the formation of platelet-activating factor acetylhydrolase (PAFAH1B) (38). The heterotrimer, PAFAH1B complex, is required in the process of neuronal migration during brain development. The above two point mutations in human Lis1 result in the lissencephaly phenotype (38, 39). These phenomena imply that Arg259 in GFRα1, which is crucial for both GFRα1 and Ret binding, may also cause clinical symptoms upon mutation in vivo.

The far-UV CD spectra and unfolding experiments show that the secondary structures and protein stabilities of the GFRα1 mutants are not significantly changed compared with wild type GFRα1. So it was verified that loss of activities of GFRα1 mutants are due to changes in binding and not simply due to unfolding of the proteins.

We demonstrated that combined residues 152NN153 and 316NS318 of GFRα1 are crucial for GDNF binding and mediating neurotrophic function of GDNF. Later individual alanine mutation of each of these residues was transfected into PC12 cells, respectively. Mutants N152A, N153A, S316A, and S318A had a major effect on GDNF-induced neurite outgrowth, indicating that they make important contributions to GDNF binding. However, N317A did not alter the response of GDNF in these PC12 cells, suggesting that it is not critical for GDNF-GFRα1 complex formation (data not shown).

In this study, we have identified a set of key amino acids in the GFRα1 receptor to be critical for functional receptor complex formation and eliciting downstream signal transduction. These residues may contribute synergistically to GDNF binding and form a functional epitope in GFRα1. Other sites that may also take part in the formation of the functional epitope of this molecule are still under investigation. Several GFRα1 mutants deficient in GDNF binding are still able to bind with Ret. This finding confirms that there are two distinct structural determinants in GFRα1 for GDNF binding and association with Ret, respectively. But a single point mutation in GFRα1 simultaneously lost its ability to bind with GDNF, and Ret implies that the two binding sites may have some overlap.

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