Structural characterization of GFRα1: Identification of ligand binding epitopes, disulfide bridges and glycosylation sites

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GFRα1 is one of four members of a family of glycosylated cysteine-rich extracellular receptors that are either secreted or membrane associated via a glycosyl-phosphatidyl-inositol (GPI) linkage. Together with the tyrosine kinase Ret, GFRα1 forms a receptor complex for glial derived neurotrophic factor (GDNF). This ligand-receptor interaction initiates the activities of GDNF in the nervous, excretory and reproductive systems. We have previously reported the mapping of a central ligand binding domain within GFRα1 and the related GFRα2, 3 and 4 (Scott and Ibanez, 2001). In this paper we report that the binding domain of GFRα1 forms a discrete core reinforced by multiple disulfide bridges. A partial tentative cystinyl map of the binding domain has been elucidated by characterization of proteolysis products. We also report two additional binding epitopes within the core-binding domain of GFRα1 by exchanges of homologous segment with GFRα2 and 4. Lastly, we have identified both N- and O-linked glycosylation sites and conclude that carbohydrate moieties in GFRα1 are not critical for its expression and interaction with its ligand.

Introduction

Glial cell line-derived neurotrophic factor (GDNF), neurturin (NTN), artemin (ART), and persephin (PSP) are potent survival promoting factors for subsets of central and peripheral neurons (Airaksinen and Saarma, 2002; Baloh et al., 2000). GDNF is also crucial for early nephrogenesis (Sariola and Saarma, 1999) and has regulatory function in sperm development (Meng et al., 2000). These molecules comprise a subclass of cystine-knot superfamily of ligands distantly related to members of the transforming growth factor β (TGFβ) superfamily. They exert their biological effects via two modes both of which are distinct from signaling through receptors for the TGFβ ligands (recently reviewed by Airaksinen and Saarma, 2002). GDNF and related ligands can interact with a class of cysteine-rich extracellular receptors collectively called GFRαs (GDNF family receptors alphas). The association of GDNF with GPI-tethered GFRα1 initiates Src-dependent signaling in membrane rafts (Poteryaev et al., 1999; Trupp et al., 1998). Alternatively, these ligands can activate a multicomponent receptor complex consisting of GFRα and the transmembrane tyrosine kinase Ret. Signaling through Ret for all the GDNF family ligands require the presence of a GFRα co-receptor. Specificity is dictated by the identity of the ligand and the isoform of GFRα involved. GDNF preferentially associates with GFRα1, while NTN, ART and PSP with GFRα2, 3 and 4, respectively (Baloh et al., 1998; Enokido et al., 1998; Jing et al., 1996; Klein et al., 1997; Scott and Ibanez, 2001).

Despite poor sequence homology with the TGF-β family of ligands, the crystal structure of GDNF (Eigenbrot and Gerber, 1997) has a striking resemblance to the structure of TGFβ1, TGFβ2, TGFβ3, BMP2, and BMP7 (Daopin et al., 1993; Griffith et al., 1996; Hinck et al., 1996; Mittl et al., 1996; Scheufler et al., 1999; Schlunegger and Grutter, 1993). The fold is characterized by a cystine knot (McDonald and Hendrickson, 1993; Murray-Rust et al., 1993) , with one cystinyl link used to form the disulfide-bridged dimer. Features shared with the monomeric structure of members of the TGFβ superfamily include two fingerlike projections comprising of β-sheets and a large α-helix protruding between two adjacent cystine knot cysteines. Surface-exposed residues and regions important for association of GDNF with GFRα1 and 2 have been identified by mutation analysis (Baloh et al., 2000; Eketjall et al., 1999).

The structure of the GFRαs and Ret are less understood. A region of the ectodomain of Ret has been modeled revealing a contiguous quartet of cadherin-like domains forming a binding site for calcium (Anders et al., 2001) but specific determinants necessary for receptor complex formation and ligand interaction are yet to be defined. Secondary structure analysis of GFRα1 to 4 revealed a high degree of conservation of predicted secondary structure elements which proved to be useful as guide in mapping the ligand binding domain (Scott and Ibáñez, 2001).

In this paper we discuss the identification of two additional binding epitopes for GDNF and NTN in GFRα1 and the identification of some cystinyl linkages within the core ligand binding domain. Furthermore, we report that the glycosylation of GFRα1 is no required for its surface expression and ligand association.

Results

Identification of ligand binding epitopes by chimera analysis of the subcentral receptor domain

We have previously mapped the ligand binding domain (LBD) for GFRα1, GFRα2, GFRα3, and GFRα4--a region averaging 200 amino acid residues--using both truncation and chimera analyses (Scott and Ibanez, 2001). We have shown that in the case of GFRα1, regions outside the LBD are not required for GDNF binding or to promote ligand activation of Ret. Distinct hydrophobic (^{211}MLF) and positively charged (^{224}RRR)

residue clusters within the LBD are critical for the interaction of GFRα1 with GDNF. Interestingly, surface -exposed hydrophobic and negatively charged determinants on GDNF have been reported as important for GFRα1 association (Eketjall et al., 1999).

In this paper we set out to identify additional ligand binding epitopes in GFRα1. Six segments of GFRα1 within the LBD were replaced with homologous regions from either GFRα2 or 4 (Fig. 1). The resulting chimeras were then compared to wild-type GFRα1 for their ability to associate with GDNF and NTN (Fig. 2). By this method, two new segments (residues 179-186 and 245-255, i.e. chimeras C, D, G, and H) were found to be importantfor ligand interaction. Chimeras C, D , and H showed a decreased ability to bind GDNF in a cross-linking assay (Fig. 2A and 3B). While the same regions are necessary for NTN binding, only chimeras D and H carrying homologous replacements with GFRα4 were able to bind NTN (Fig. 2B and 3B). The remaining chimeras made were able to bind GDNF comparable to wild-type GFRα1 (cross-linking analysis for chimeras I to P are not shown).

The ligand binding domain of GFRα1 forms a discrete core unit reinforced by disulfide bridges

Twenty-eight cysteines are conserved among the GFRα receptors, and two additional ones are conserved only between GFRα1 and GFRα2, most of which are likely to form disulfide bridges. As a first step in the identification of potential cystinyl bonds, we digested GFRα1 with cyanogen bromide (CNBr) and fractionated the peptide fragments by high performance liquid chromatography (HPLC) on a reversed phase column. For this purpose, we used a commercial fusion protein between the rat GFRα1 (first 445 residues) and human immunoglobulin (IgG_1) Fc chain (residues Pro100 to Lys330). The acidic conditions used during CNBr digestion allowed for the preservation of cystinyl linkages.

CNBr, which specifically cleaves after methionine, generated a hydrophobic peptide fraction which after N-terminal Edman sequencing was found to consist of four contiguous peptides (thick arrow in Fig. 3A): Tyr108-Met182, Ser183-Met211, Leu212-Met297, and Thr298-Met353. Interestingly, these peptides span the LBD of GFRα1 (Fig. 3B) (Scott and Ibáñez, 2001). Elimination of sulfhydryl bonds in the CNBr digest by either reduction with dithiothreitol or performic acid oxidation led to the disappearance of this HPLC peak. This suggested that the LBD forms a discrete core that is multiply reinforced by cystinyl bonds.

The CNBr fraction spanning the LBD of GFRα1 was further digested with trypsin for identification of the position of the sulfhydryl bonds within this region. Peptides containing cysteines were identified by comparing HPLC profiles of tryptic digests before and after chemical reduction. We have so far been able to identify, four well resolved chromatographic peaks using this approach (see Fig. 4A). N-terminal Edman sequencing of these HPLC fractions confirmed that they included cysteine-containing peptides. Cysteine residues located within co-eluting tryptic peptides were tentatively assigned as being disulfide-linked. From this analysis we have deduced the following partial cystinyl map of the LBD of GFRα1 (Fig. 4B): Cys154-221; Cys-161-Cys167; Cys178-Cys233; Cys214-Cys216; Cys243-Cys250; and Cys267-Cys285.

Previous deletion analysis on GFRα1 and the existence of N-terminally truncated isoforms of GFRα2 suggest that the N- and C-terminal domains flanking the LBD are not disulfide-linked (Scott and Ibáñez, 2001). Two cysteines are found in the C-terminal domain of mature GFRα1 and these are also conserved in GFRα2. We mutated these cysteines (C395S and C414S) to assess whether they are linked to each other. We anticipated that generation of a free thiol by mutagenesis would disrupt the normal cystinyl linkages and thus impair the expression of the receptor. This notion is consistent with our failure to express an N-terminally truncated mutant of GFRα1 (ΔN84) which has an odd-number of cysteine residues (Scott and Ibáñez, 2001). Surprisingly, the mutations C395S and C414S had no effect on GFRα1 expression (Fig. 4C), and thus do not allow us to conclusively establish whether they are engaged in the formation of dislfide bridges.

Glycosylation of GFRα1

The predicted mass for full-length, HA-tagged GFRα1 (discounting the N-terminal signal peptide and C-terminal peptide sequences past the putative GPI anchorage site) is 47 kD, yet empirical masses as high as 60 kD are generally observed upon receptor expression in fibroblast and neuronal cells (Scott and Ibáñez, 2001). Enzymatic removal of N-glycan moeities by PNGase F (PNGF) treatment revealed that N-glycosylation accounted for as much as 10 kD of the mass whereas O-glycosylation accounted for an additional 5 kD (Fig. 5A). We set out to investigate by mutagenesis which of the candidate glycosylation sites in GFRα1 were actually contributing to its postranslational modification.

Three N-glycosylation sites are predicted for GFRα1: Asn59, Asn347, and Asn406. The receptor mutant N347A, showed a mass shift, albeit only partial, confirming that this site is glycosylated. We then built the double mutant N59A/N347A which also showed a mass shift, verifying that Asn59 is an additional site for attachment of carbohydrate moieties. PNGF treatment of the receptor double mutant N59A/N347A, however, showed a further decrease in mass thus suggesting that Asn406 is also likely to be a third N-glycosylation site.

O-glycosylation on the other hand is predicted to be clustered in a threonine-rich segment of GFRα1 downstream of the LBD (threonine residues 362, 363, 364, 366, 367, 368, and 369). To assess its possible postranslational modification, the whole segment was deleted (ΔOG: Thr362 to Thr369). The ΔOG mutant protein migrated as a single protein band in gels, confirming the presence of O-glycosylation within the deleted segment (Fig. 5A). A similar pattern was also observed for the deletion mutant ΔC21 (lacking residues 348 to 369), encompassing the ΔOG deletion with an additional disruption of the N-glycosylation site at Asn347.

We have also characterized a receptor construct incorporating both the N59A and ΔC21 mutations. The product of the resulting construct was still PNGF-sensitive, thus confirming the existence of an additional N-glycosylation site. To assess whether this N-glycosylation site was within the LBD of GFRα1, we characterized the PNGF sensitivity of the truncated molecule ΔN144ΔC55 (deletion of N-terminal residues 1-144 and C-terminal segment 356-421; see also Scott and Ibáñez, 2001). The mutant receptor ΔN144/ΔC55 was N-glycosylated based on mass shift upon PNGF digestion. Incorporation of the mutation N347A (mutant ΔN144/ΔC55/N347A), led to complete loss of PNGF sensitivity suggesting that only Asn347 is utilized for N-glycosylation within the LBD. Asn406 is likely the third glycosylation site based on predictions.

In crosslinking assays, none of the mutations found to affect receptor glycosylation had any effect on the ability of GFRα1 to interact with GDNF (Fig. 5B). Although the glycosylation of Asn406 is yet to be verified, we infer that it is unlikely to influence ligand binding, since the truncation mutant ΔN144/ΔC55 (which lacks Asn406) is still capable of binding ligand (Scott and Ibáñez, 2001). We conclude that glycosylation does not affect the surface expression orligand binding properties of GFRα1.

Discussion

Threading and homology analyses (Bates et al., 2001; Kelley et al., 2000) have so far been unssuccesfull in assigning three-dimensional folds with reasonable significance for any of the GFRα receptors. Although GDNF is structurally very similar to members of the TGF-β superfamily of ligands, TGF-β receptors are completely different to GFRαs both in their domain structure and functionality. The small ectodomains of the high affinity type II receptors for the TGF-β-type ligands lack sequence homology with any of the domains of the GFRα receptors. Moreover, the crystal structures of the ligand interacting domains of the type II receptors for activin and TGF-β3 show a predominance of β strands (Greenwald et al., 1999; Hart et al., 2002) which are in contrast with the high α-helical content of the predicted secondary structure of the GFRα receptors. Intriguingly, and despite these differences, both GDNF and TGFβ molecules interact with their respective receptors via analogous residues in the finger regions of these ligands (Baloh et al., 2000; Eketjall et al., 1999; Hart et al., 2002).

The characterization of CNBr-fragmentation of GFRα1 confirms our initial report that the ligand binding surface of GFRα family of receptors, forms a discrete core which can be dissociated from the the rest of the molecule without loss of activity. Our present resuts indicate that the ligand binding domain is reinforced by multiple intradomain disulfide bridges. Analysis of truncated mutants of GFRα1 (Scott and Ibáñez, 2001) have suggested that intradomain disulfide linkage may also exists in the N-terminal domain.

In our analysis we have identified two new ligand binding epitopes in GFRα1 which are relevant for its interaction with both GDNF and NTN in residues 179-186 and 245-255, respectively. The replacement of the segment comprised by residues 179-186 with the homologous section from GFRα2 or GFRα4 (chimeras C and D, respectively), compromised interaction with GDNF. However, only the exchange with GFRα4 sequences affected NTN binding. Segment 179-186 is shorter by one residue relative to the corresponding regions in GFRα2 and GFRα4. The homologous section of GFRα3 is notably smaller. Moreover, residue charge distributions are also unique within this region: GFRα4 is notably more basic than either GFRα1 or GFRα2. In the case of residues 245-255 of GFRα1, replacement with the corresponding sequence from GFRα4, but not with that of GFRα2, reduced binding of both GNDF and NTN. This segment has the same length in all GFRα receptors but a more acidic charge compositon in GFRα4 than in the other GFRαs. Both receptor epitopes map within predicted loops (Fig. 3B) (Scott and Ibáñez, 2001). Interestingly, our preliminary mapping of disulfide bonds in GFRα1, suggests that the four ligand binding determinants identified so far are drawn closer by the cystinyl bridges Cys154-Cys221 and Cys178-Cys233 in the three dimensional structure of GFRα1 (see Fig. 4B).

Finally, we have extended the structural characterization of GFRα1 with the identification of its glycosylation sites. While the existence of N-glycosylation was verified in both the N-terminal (Asn59) and ligand binding (Asn347) region and predicted at the carboxy tail of the receptor (Asn406), O-glycosylation was found to be clustered within a threonine-rich region downstream of the ligand binding domain. Removal of these sites by mutagenesis revealed that glycosylation of GFRαs does not appear to have structural significance . The glycosylation mutants of GFRα1 we have analyzed are expressed at levels comparable to the wild-type receptor and are all competent in binding GDNF. GFRα1 is known to be secreted as as soluble receptor (Paratcha et al., 2001; Worley et al., 2000) and one possible role of its glycosylation may be the facilitation of its of interaction withextracellular matrix components. Interaction of GFRα1 with matrix components could play a role in GDNF-induced chemoattraction during nervous system development (Young et al., 2001). An alternative role for glycosylation may be in the polarized expression and secretion of GFRαs. Glycosylation has been identified as an apical sorting signal in polarized epithelial cells (Benting et al., 1999; Naim et al., 1999; Scheiffele et al., 1995).

The determination of the complete disulfide structure of GFRα1 is ongoing. Unequivocal assignments of cystinyl bonds is planned to be performed by mass spectrometry analysis. The results presented here indicate that key determinants for ligand binding are spatially close. The apparent lackk of importance of glycosylation for both receptor expression and function could be beneficial in large-scale expression of the GFRα1 for more refined structural characterization by, for example X-ray crystallography. On the basis of secondary structural topology and the existence of multiple disulfide

linkages, we expect that GFRα1 and the related GFRα receptors to constitute a novel three-dimensional fold.

Experimental Procedures

DNA constructs and mutagenesis

Hemagglutinin (HA)-tagged rat GFRα1, rat GFRα2 and chicken GFRα4 expression constructs were described previously (Scott and Ibáñez, 2001). Deletions in GFRα1 and segmental exchanges within the ligand binding domain (from amino acid residues 179 and 285) with homologous regions of either GFRα2 or 4, were all accomplished by Quick Change mutagenesis (Stratagene). Candidate glycosylation sites were predicted using ScanProsite and (Falquet et al., 2002; Hansen et al., 1998; Hofmann et al., 1999) and were mutated similarly.

Cell culture and transfection

COS7 were grown in DMEM-based medium (Invitrogen) with 10% fetal bovine serum, 1mM glutamine and gentamicin in a 5% CO_2/95% O_2 incubator at 37°C. Cells were transiently transfected with branched polyethyleneimine (25 kD, Aldrich).

Ligand crosslinking assay

Radioiodination of GDNF and ligand-receptor crosslinking assay has been described earlier (Scott and Ibáñez, 2001). Briefly, COS7 cells transiently transfected with various receptor constructs were crosslinked to [^{125}I]GDNF at 4°C using bis(succinimidyl) suberate (BS^3) reagent (Pierce). Cell lysates were processed for autoradiography and immunoblotting.

Characterization of glycosylation

GFRα1 constructs mutated and putative glycosylation sites were expressed in COS7 cells transiently. Cell lysates were either left untreated or digested with PNGaseF (New England Biolabs) to remove N-glycans, and samples were analyzed in protein immunoblots. Extent of glycosylations and effects of introduced mutations were assessed by the heterogeneity of HA-tagged protein bands and mobility shifts upon PNGaseF digestion.

Protein gels and immunoblotting

Cells were lysed in immunoprecipitation 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; and 2 mM Na_3VO_4). Immunoprecipitation for expressed receptor and ligand-receptor complexes was done using an anti-HA monoclonal antibody (Covance). Protein blots were made on PVDF membrane (Amersham Pharmacia). Immunoblot detection using alkaline-phosphatase based chemifluorescence and autoradiography were carried out using a Storm phosphoimager (Amersham Pharmacia).

Mapping of disulfide bridges

A commercial preparation of GFRα1 fused N-terminally to the Fc segment of human immunoglobulin (GFRα1-Fc, R&D Systems) was desalted by gel filtration using 30% acetic acid and lyophilized prior to digestion. Cyanogen bromide (CNBr) cleavage was done overnight in 70% aqueous formic acid. CNBr-digests were separated on a 2.1 by 150 mm C8 reverse phase column (Vydac) using a discontinous solvent gradient from 100% solvent A (0.1% aqueous trifluoroacetic acid, TFA) to 100% solvent B (0.085% TFA/20% water/80% CH_3CN) over 72 min at a flow rate of 200 μL/min. Tryptic digestion of CNBr peaks was done overnight at 37°C in a 100-μL scale using 0.5 μg trypsin (Promega) with the dried CNBr digests redissolved in 50 mM NH_4CO_3/10% CH_3CN plus 2.5 mM iodoacetamide. The tryptic fragments were fractionated using a similar chromatographic gradient used for the CNBr digests but on a 2.1 by 25 mm monomeric C18 column (Vydac). To identify potential cystinyl-containing peptides, chromatograms were compared against an aliquot of the tryptic digest that has been reduced. Reduction of tryptic fragments was done using 20 mM tris(2-carboxyethylphosphine) hydrochloride (TCEP) in 10% aqueous acetic acid for 2.5 h at 37°C.

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References

Airaksinen, M. S., and Saarma, M. (2002). The GDNF family: signalling, biological functions and therapeutic value. Nat Rev Neurosci *3*, 383-94.

Anders, J., Kjar, S., and Ibanez, C. F. (2001). Molecular modeling of the extracellular domain of the RET receptor tyrosine kinase reveals multiple cadherin-like domains and a calcium-binding site. J Biol Chem *276*, 35808-17.

Baloh, R. H., Tansey, M. G., Johnson, E. M., Jr., and Milbrandt, J. (2000). Functional mapping of receptor specificity domains of glial cell line-derived neurotrophic factor (GDNF) family ligands and production of GFRalpha1 RET-specific agonists. J Biol Chem *275*, 3412-20.

Baloh, R. H., Tansey, M. G., Lampe, P. A., Fahrner, T. J., Enomoto, H., Simburger, K. S., Leitner, M. L., Araki, T., Johnson, E. M., Jr., and Milbrandt, J. (1998). Artemin, a novel member of the GDNF ligand family, supports peripheral and central neurons and signals through the GFRalpha3-RET receptor complex. Neuron *21*, 1291-302.

Bates, P. A., Kelley, L. A., MacCallum, R. M., and Sternberg, M. J. (2001). Enhancement of protein modeling by human intervention in applying the automatic programs 3D-JIGSAW and 3D-PSSM. Proteins *Suppl 5*, 39-46.

Benting, J. H., Rietveld, A. G., and Simons, K. (1999). N-Glycans mediate the apical sorting of a GPI-anchored, raft-associated protein in Madin-Darby canine kidney cells. J Cell Biol *146*, 313-20.

Daopin, S., Li, M., and Davies, D. R. (1993). Crystal structure of TGF-beta 2 refined at 1.8 A resolution. Proteins *17*, 176-92.

Eigenbrot, C., and Gerber, N. (1997). X-ray structure of glial cell-derived neurotrophic factor at 1.9 A resolution and implications for receptor binding. Nat Struct Biol *4*, 435-8.

Eketjall, S., Fainzilber, M., Murray-Rust, J., and Ibanez, C. F. (1999). Distinct structural elements in GDNF mediate binding to GFRalpha1 and activation of the GFRalpha1-c-Ret receptor complex. Embo J *18*, 5901-10.

Enokido, Y., de Sauvage, F., Hongo, J. A., Ninkina, N., Rosenthal, A., Buchman, V. L., and Davies, A. M. (1998). GFR alpha-4 and the tyrosine kinase Ret form a functional receptor complex for persephin. Curr Biol *8*, 1019-22.

Falquet, L., Pagni, M., Bucher, P., Hulo, N., Sigrist, C. J., Hofmann, K., and Bairoch, A. (2002). The PROSITE database, its status in 2002. Nucleic Acids Res *30*, 235-8.

Greenwald, J., Fischer, W. H., Vale, W. W., and Choe, S. (1999). Three-finger toxin fold for the extracellular ligand-binding domain of the type II activin receptor serine kinase. Nat Struct Biol *6*, 18-22.

Griffith, D. L., Keck, P. C., Sampath, T. K., Rueger, D. C., and Carlson, W. D. (1996). Three-dimensional structure of recombinant human osteogenic protein 1: structural paradigm for the transforming growth factor beta superfamily. Proc Natl Acad Sci U S A *93*, 878-83.

Hansen, J. E., Lund, O., Tolstrup, N., Gooley, A. A., Williams, K. L., and Brunak, S. (1998). NetOglyc: prediction of mucin type O-glycosylation sites based on sequence context and surface accessibility. Glycoconj J *15*, 115-30.

Hart, P. J., Deep, S., Taylor, A. B., Shu, Z., Hinck, C. S., and Hinck, A. P. (2002). Crystal structure of the human TbetaR2 ectodomain--TGF-beta3 complex. Nat Struct Biol *9*, 203-8.

Hinck, A. P., Archer, S. J., Qian, S. W., Roberts, A. B., Sporn, M. B., Weatherbee, J. A., Tsang, M. L., Lucas, R., Zhang, B. L., Wenker, J., and Torchia, D. A. (1996). Transforming growth factor beta 1: three-dimensional structure in solution and comparison with the X-ray

structure of transforming growth factor beta 2. Biochemistry *35*, 8517-34.

Hofmann, K., Bucher, P., Falquet, L., and Bairoch, A. (1999). The PROSITE database, its status in 1999. Nucleic Acids Res *27*, 215-9.

Jing, S., Wen, D., Yu, Y., Holst, P. L., Luo, Y., Fang, M., Tamir, R., Antonio, L., Hu, Z., Cupples, R., Louis, J. C., Hu, S., Altrock, B. W., and Fox, G. M. (1996). GDNF-induced activation of the ret protein tyrosine kinase is mediated by GDNFR-alpha, a novel receptor for GDNF. Cell *85*, 1113-24.

Kelley, L. A., MacCallum, R. M., and Sternberg, M. J. (2000). Enhanced genome annotation using structural profiles in the program 3D-PSSM. J Mol Biol *299*, 499-520.

Klein, R. D., Sherman, D., Ho, W. H., Stone, D., Bennett, G. L., Moffat, B., Vandlen, R., Simmons, L., Gu, Q., Hongo, J. A., Devaux, B., Poulsen, K., Armanini, M., Nozaki, C., Asai, N., Goddard, A., Phillips, H., Henderson, C. E., Takahashi, M., and Rosenthal, A. (1997). A GPI-linked protein that interacts with Ret to form a candidate neurturin receptor. Nature *387*, 717-21.

McDonald, N. Q., and Hendrickson, W. A. (1993). A structural superfamily of growth factors containing a cystine knot motif. Cell *73*, 421-4.

Meng, X., Lindahl, M., Hyvonen, M. E., Parvinen, M., de Rooij, D. G., Hess, M. W., Raatikainen-Ahokas, A., Sainio, K., Rauvala, H., Lakso, M., Pichel, J. G., Westphal, H., Saarma, M., and Sariola, H. (2000). Regulation of cell fate decision of undifferentiated spermatogonia by GDNF. Science *287*, 1489-93.

Mittl, P. R., Priestle, J. P., Cox, D. A., McMaster, G., Cerletti, N., and Grutter, M. G. (1996). The crystal structure of TGF-beta 3 and comparison to TGF-beta 2: implications for receptor binding. Protein Sci *5*, 1261-71.

Murray-Rust, J., McDonald, N. Q., Blundell, T. L., Hosang, M., Oefner, C., Winkler, F., and Bradshaw, R. A. (1993). Topological similarities in TGF-beta 2, PDGF-BB and NGF define a superfamily of polypeptide growth factors. Structure *1*, 153-9.

Naim, H. Y., Joberty, G., Alfalah, M., and Jacob, R. (1999). Temporal association of the N- and O-linked glycosylation events and their implication in the polarized sorting of intestinal brush border sucrase-isomaltase, aminopeptidase N, and dipeptidyl peptidase IV. J Biol Chem *274*, 17961-7.

Paratcha, G., Ledda, F., Baars, L., Coulpier, M., Besset, V., Anders, J., Scott, R., and Ibanez, C. F. (2001). Released GFRalpha1 potentiates downstream signaling, neuronal survival, and differentiation via a novel mechanism of recruitment of c-Ret to lipid rafts. Neuron *29*, 171-84.

Poteryaev, D., Titievsky, A., Sun, Y. F., Thomas-Crusells, J., Lindahl, M., Billaud, M., Arumae, U., and Saarma, M. (1999). GDNF triggers a novel ret-independent Src kinase family-coupled signaling via a GPI-linked GDNF receptor alpha1. FEBS Lett *463*, 63-6.

Sariola, H., and Saarma, M. (1999). GDNF and its receptors in the regulation of the ureteric branching. Int J Dev Biol *43*, 413-8.

Scheiffele, P., Peranen, J., and Simons, K. (1995). N-glycans as apical sorting signals in epithelial cells. Nature *378*, 96-8.

Scheufler, C., Sebald, W., and Hulsmeyer, M. (1999). Crystal structure of human bone morphogenetic protein-2 at 2.7 A resolution. J Mol Biol *287*, 103-15.

Schlunegger, M. P., and Grutter, M. G. (1993). Refined crystal structure of human transforming growth factor beta 2 at 1.95 A resolution. J Mol Biol *231*, 445-58.

Scott, R. P., and Ibanez, C. F. (2001). Determinants of ligand binding specificity in the glial cell line-derived neurotrophic factor family receptor alpha S. J Biol Chem *276*, 1450-8.

Trupp, M., Raynoschek, C., Belluardo, N., and Ibanez, C. F. (1998). Multiple GPI-anchored receptors control GDNF-dependent and independent activation of the c-Ret receptor tyrosine kinase. Mol Cell Neurosci *11*, 47-63.

Worley, D. S., Pisano, J. M., Choi, E. D., Walus, L., Hession, C. A., Cate, R. L., Sanicola, M., and Birren, S. J. (2000). Developmental regulation of GDNF response and receptor expression in the enteric nervous system. Development *127*, 4383-93.

Young, H. M., Hearn, C. J., Farlie, P. G., Canty, A. J., Thomas, P. Q., and Newgreen, D. F. (2001). GDNF is a chemoattractant for enteric neural cells. Dev Biol *229*, 503-16.

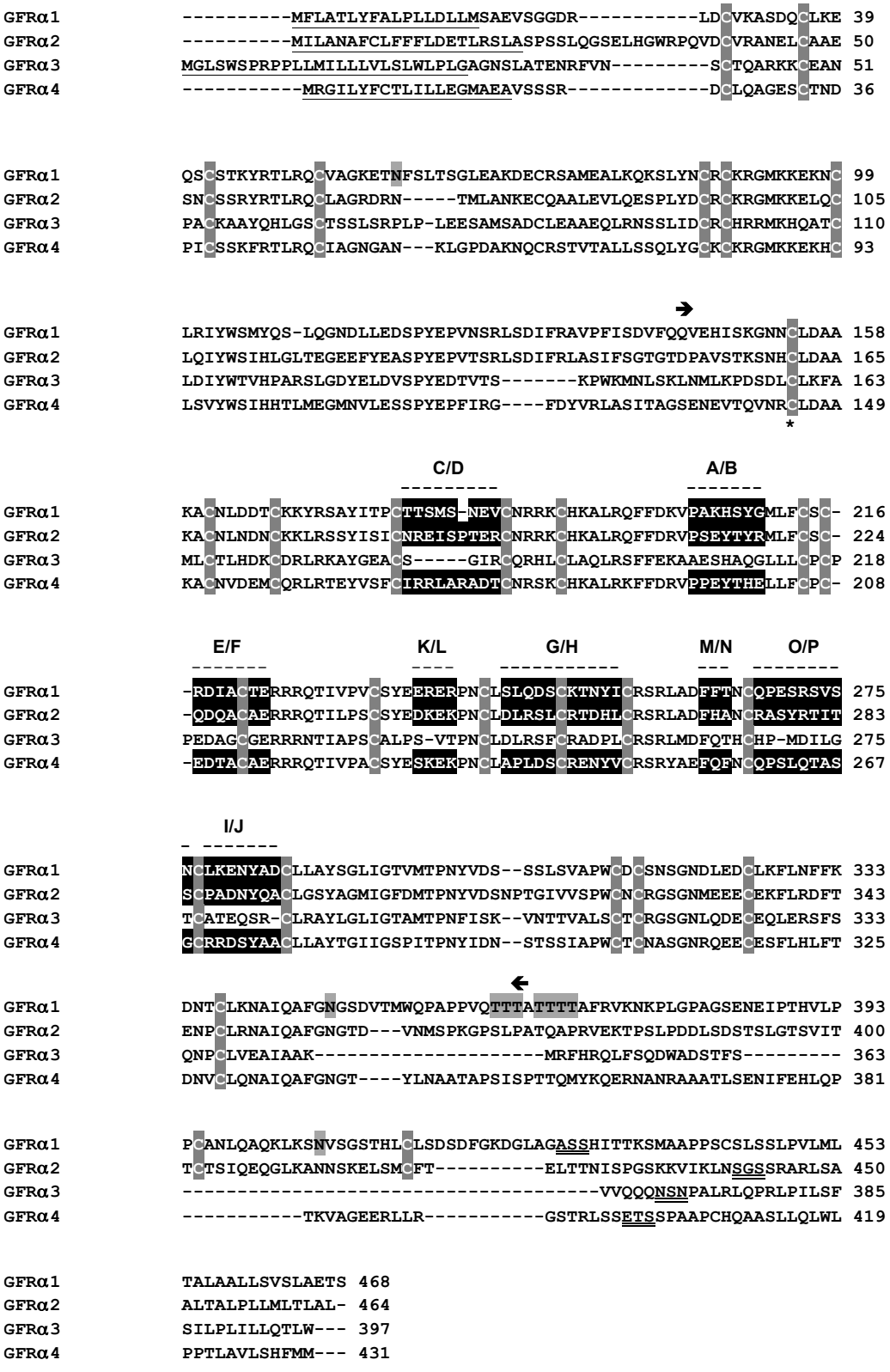

Figure 1. Sequence alignment of members of the GFRα family of receptors. Primary sequences of GFRα1 (rat), GFRα2 (rat), GFRα3 (mouse), and GFRα4 (chicken) were aligned using ClustalW. The ligand binding domain is demarcated by forward and reverse arrows above the alignments. Segments within the ligand binding domain of GFRα1 that were replaced with homologous regions from either GFRα2 or GFRα4 are shaded in black. As indicated above the alignments, chimeras built between GFRα1 and GFRα2 were given indicated names A, C, E, G, I, K, M and O while those built between GFRα1 and GFRα4 were named B, D, F, H, J, L, N, and P. Conserved cysteines written as white characters shaded in grey whereas predicted glycosylation sites in GFRα1 are in black characters shaded in grey. N-terminal signal peptide sequences are underlined once while the predicted C-terminal protease cleavage site for GPI-anchorage are doubly underlined.

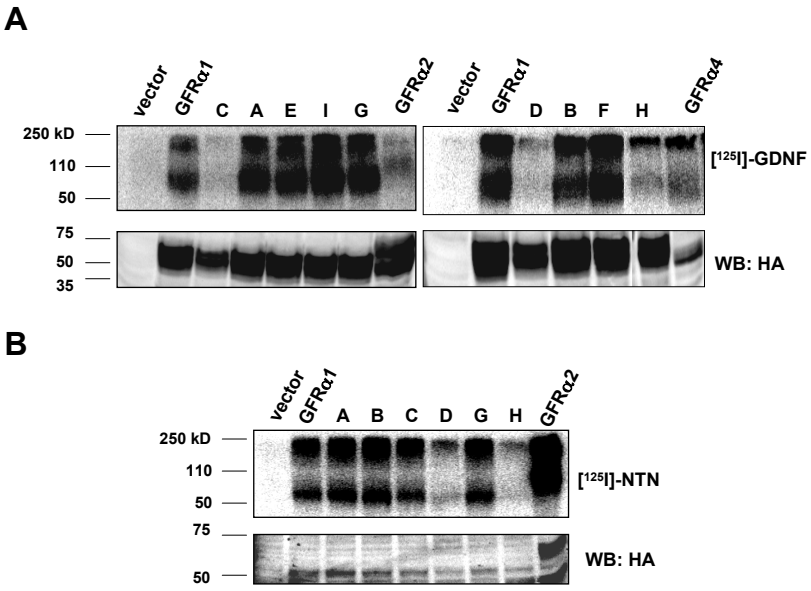

Figure 2. Ligand binding properties of subcentral domain chimeras. *(A) GDNF crosslinking assay. (B) NTN crosslinking assay.* Receptor constructs were expressed transiently in COS7 cells and crosslinked covalently with iodinated ligands. Upper panels show autoradiographs of ligand-receptor complexes while the lower panels show receptor expression levels as determined by protein immunoblotting using an anti-HA antibody.

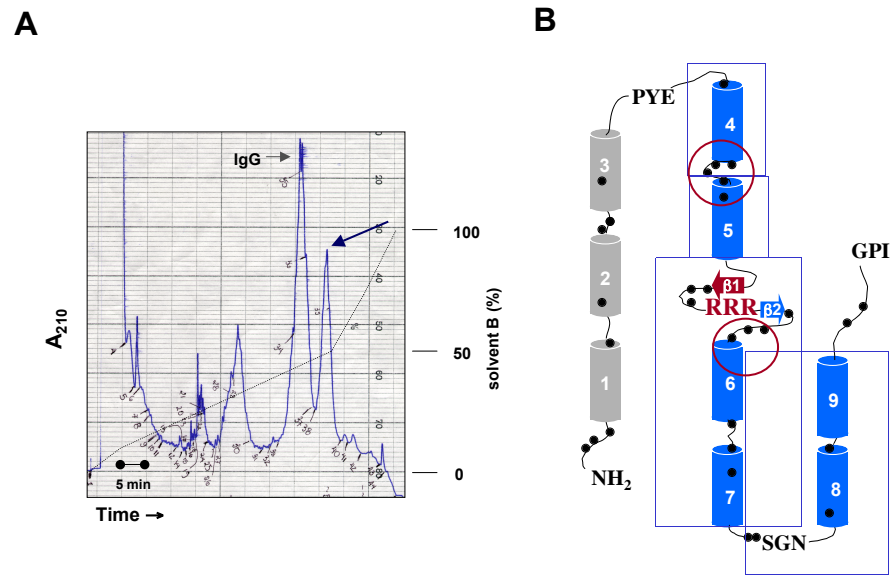

Figure 3. The ligand binding domain is structurally discrete and likely reinforced by multiple disulfide bridges. *(A) Proteolytic products of the ligand binding domain are physically linked.* Products of the cleavage of GFRα1-Fc fusion protein with CNBr were separated and collected by reversed-phase HPLC, and sequenced by Edman degradation. The hydrophobic fraction containing four peptides spanning the ligand binding domain of GFRα1 is marked by a thick arrow. The immunoglobulin fraction containing most of the Fc tag is marked with a small arrow (IgG).HPLC conditions are described in the experimental section. Solvent ramp is indicated by dotted line segments. *(B) Ligand binding determinants in GFRα1.* Shown is a shematic representation of the predicted secondary structure of GFRα1. Linked CNBr-peptides described in (A) are marked by dotted blue boxes. Ligand binding determinants found in this study are encircled in red while two other epitopes identified previously (Scott and Ibáñez, 2001) are also marked in red . Predicted helices are shown as cylinders: grey in the N-terminal domain and blue in the ligand binding domain. Beta strands are shown as thick arrows within the ligand binding domain. Conserved cyteines are indicated as thick dots.

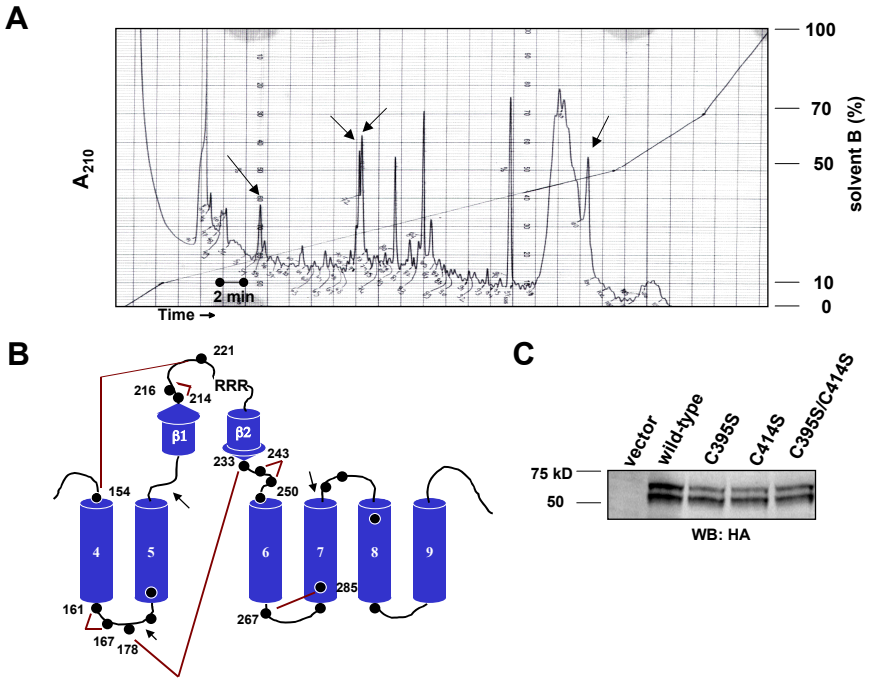

Figure 4. Mapping of the disulfide structure of GFRα1. (A) Chromatographic profile of the tryptic fragments of the ligand binding domain. The ligand binding domain separated by a preliminary CNBr-cleavage was digested with trypsin. Putative cystinyl-containing peptides were identified by comparison with the HPLC profile of a TECP-reduced aliquot of the tryptic digest . Only the profile of the unreduced aliquot is shown. Fractions with identified Cys-containing peptides are indicated by arrows. (B) Partial disulfide structure within the ligand binding domain of GFRα1. (C) Mutation of C-terminal domain cysteines do not affect expression of GFRα1. Cys-395 and -415, beyond the ligand binding domain were mutated singly or in combination to serine. The resulting mutant constructs are expressed comparably as wild-type GFRα1 in fibroblast cells.

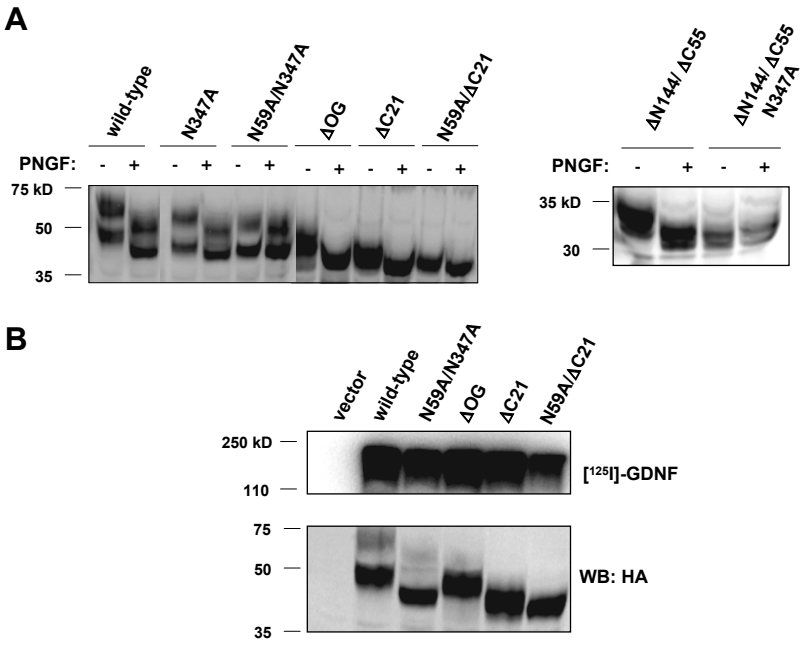

Figure 5. Glycosylation of GFRα1. *(A) Mapping of glycosylation sites in GFRα1.* Putative glycosylation mutants of GFRα1 were compared with wild-type GFRα1 for mass shifts after PNGF treatment. Shown are protein blots probed with anti-HA antibody. *(B) Glycosylation deficiency in GFRα1 does not impair ligand binding.* Glycosylation mutants were transiently expressed in COS7 cells and crosslinked to radioiodinated GDNF. Upper panels shows an autoradiograph of the crosslinked ligand-receptor complex while the lower panel shows the expression levels of the receptor constructs.