Identification of a Surface for Binding to the GDNF-GFRα1 Complex in the First Cadherin-like Domain of RET

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The RET receptor tyrosine kinase is activated by binding to a ligand complex formed by a member of the glial cell line-derived neurotrophic factor (GDNF) family of neurotrophic factors bound to its cognate GDNF-family receptor-α (GFRα) glycosylphosphatidylinositol-linked co-receptor. Molecular modeling studies of the extracellular domain of RET (RET-EC) have revealed the existence of four cadherin-like domains (CLD1–4) followed by a cysteine-rich domain. Cross-linking experiments have indicated that the RET-EC makes direct contacts with both the GDNF ligand and GFRα1 molecule in the complex, although it has low or no detectable affinity for either component alone. We have exploited sequence and functional divergences between the ectodomains of mammalian and amphibian RET molecules to map binding and functional divergences between the ectodomains of either component alone. We have exploited sequence and functional divergences between the ectodomains of mammalian and amphibian RET molecules to map binding and functional divergences between the ectodomains of either component alone.

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growth factor-β proteins with their cognate type II receptors (15). The major determinant of ligand binding in GFRα molecules has been localized to the most conserved region of the molecule, a central domain predicted to contain four conserved α-helices and two short β-strands (12). Distinct hydrophobic and positively charged residues in this central region were required for the binding of GFRα1 to GDNF (12). Because the RET<sub>EC</sub> is unable to bind members of the GDNF family directly, and only weakly to GFRα molecules (16), this receptor is likely to interact with a composite surface formed by residues from both GDNF and GFRα molecules. However, the regions and residues in RET<sub>EC</sub> that participate in these interactions have remained unknown.

The overall molecular architecture of the RET<sub>EC</sub> was recently elucidated using a bioinformatics approach (17). In that study, it was found that the RET<sub>EC</sub> comprises four N-terminal domains with similarity to classical cadherin molecules, so-called cadherin-like domains or CLDs, followed by a C-terminal cysteine-rich domain. Multiple alignments indicate that the RET<sub>EC</sub> from a number of different species, including human, mouse, chick, frog, fish, and fly, appear to conform to this organization (17). The highest degree of sequence similarity between the RET<sub>EC</sub> and cadherins is found in and around a highly conserved calcium binding site present between CLD2 and CLD3 but, unlike classical cadherins, absent between all other RET<sub>EC</sub> subdomains (17).

In the present study, we have investigated the location and biochemical characteristics of ligand binding determinants in the human RET<sub>EC</sub>. For this purpose, we have employed homologue-scanning mutagenesis, taking advantage of the inability of the Xenopus RET<sub>EC</sub> to interact with complexes between GDNF family ligands and GFRα molecules of mammalian origin, despite its overall structural similarity to the human RET<sub>EC</sub>.

**MATERIALS AND METHODS**

**DNA Constructs**—All expression constructs were generated in the pSecTag2AHA system (18). The cDNA encoding the mature part of the RET<sub>EC</sub> was amplified by PCR and cloned into the NotI sites of the pSecTag2AHA vector. The chimeric constructs were generated by splicing by overlap extension (19). The integrity of the cloning junctions of all constructs were confirmed by automated DNA sequencing. The regions targeted for mutation by en-block mutagenesis were identified using the GETAREA 1.1 software (20) (www.scbio.utmb.edu/getarea/area_man.html) with the coordinates from the modeled CLD (1–3) as input (17).

**Transfection and Selection of Stable CHO Cell Lines**—Chinese hamster ovary (CHO) cells were maintained in a humid atmosphere of 5% CO<sub>2</sub> in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 2% fetal calf serum, 50 μg/ml gentamycin and 10% fetal bovine serum. Freshly split CHO cells were transfected using FuGENE 6 with 800 ng of a fusion protein formed by rat GFRα1-Fc, R&D Systems (21) in PBS. In some experiments, the related ligand NTN (human) and the Fc fusion of its cognate GFRα2 receptor (from rat) were also used. After 5 min of incubation at room temperature, the protein solution was added to the wells of an enzyme-linked immunosorbent assay plate (MaxiSorp, Nunc). The following day, the wells were rinsed by PBS and blocked with 2% skimmed milk powder in TBS (2% MTBS) for 1 h at room temperature. Equal amounts of RET<sub>EC</sub> mutants were added to the GDNF-GFRα1-Fc coated wells and to wells only coated with 2% MTBS. The binding was allowed to proceed for 1 h at room temperature. The washing was performed with TBS with 0.1% Tween 20 three times and TBS three times. Following washing, bound RET<sub>EC</sub> molecules were detected by a monoclonal anti-HA antibody at a 1:2000 dilution in 2% MTBS. Finally, the monoclonal anti-HA antibody was detected with an anti-mouse horseradish peroxidase-conjugated antibody (DAKO) at a 1:10000 dilution in 2% MTBS. The reaction was developed by addition of 3,3’3,5-tetramethyl benzidine (TMB) substrate according to the instructions of the manufacturer (Pierce).

**Native Deglycosylation of Human RET<sub>EC</sub> and Binding Analysis**—To examine the influence of the N-linked carbohydrates attached to the ectodomain of RET, the carbohydrates were enzymatically removed under native conditions. Briefly, 1 μg of semipurified HA-tagged RET<sub>EC</sub> was deglycosylated by incubation with 20 units of PNGase F (Roche Applied Science) at 37 °C for 4 h. Half of the reaction was applied for binding experiments as described above. The remaining portion was taken for Western blot analysis to verify the removal of N-linked carbohydrates.

**RESULTS**

**Preferential Interaction of the Mammalian GDNF-GFRα1 Ligand Complex with Human, but Not Xenopus, RET<sub>EC</sub>**—To evaluate the functional capabilities of wild-type and mutant RET<sub>EC</sub> molecules, we have developed a solid-phase binding assay using immobilized human GDNF, rat GFRα1, or the GDNF-GFRα1 complex as target ligands (18). Recombinant RET<sub>EC</sub> molecules were produced as epitope-tagged, soluble proteins in serum-free supernatants of stable transfected CHO cell lines as described previously (18). As shown in Fig. 1A, human RET<sub>EC</sub> was able to detect the GDNF-GFRα1 complex with an EC<sub>50</sub> of ~0.2 nM, a value comparable with the binding affinity reported previously using cell-based cross-linking binding assays (10). The human RET<sub>EC</sub> did not interact with either GDNF or GFRα1 alone (Fig. 1A), in agreement with previous observations. Similar to cell-based binding assays (17, 21, 22), the interaction between the human RET<sub>EC</sub> and the GDNF-GFRα1 complex in our solid-phase binding assay was dependent on Ca<sup>2+</sup>, as it was totally abolished in the presence of 1 mM of the Ca<sup>2+</sup>-specific chelator EGTA (Fig. 1B). Based on its structural role in the cadherin molecule, the binding of Ca<sup>2+</sup> ions to the CLD2/CLD3 interface of the RET<sub>EC</sub> is thought to rigidify the relative orientations of these two cadherin-like domains in RET<sub>EC</sub> (23, 24). The requirement of Ca<sup>2+</sup> for the ability of the RET<sub>EC</sub> to interact with the GDNF-GFRα1 complex suggests that residues located on both sides of the Ca<sup>2+</sup>-binding site between CLD2 and CLD3 may contribute to ligand binding. Despite its overall structural similarity to human RET<sub>EC</sub> (17), the Xenopus RET<sub>EC</sub> has only 45% amino acid identity to its human counterpart, indicating a significant level of sequence divergence between the two species. In contrast, the intracellular domains of human and Xenopus RET display linked immunosorbent assay using anti-HA antibodies as capture tools and a polyclonal rabbit anti-RET<sub>EC</sub> antibody (18) as detection reagent.

**Deglycosylation Assays**—To verify the structural integrity (and consequent ability of the molecules to pass the “quality control checkpoint” of the endoplasmic reticulum) of the RET domain chimeras and fine-tuned mutants, the chimeric RET<sub>EC</sub> mutants were subjected to deglycosylation assays using endoglycosidase H (Endo H) and peptide:N-glycosidase F (PNGase F) deglycosylases. Typically, 50 μl of conditioned medium was either treated with deglycosidase or left untreated. The deglycosylation was carried out according to instructions of the manufacturer (New England Biolabs).

**Binding Assays**—The binding experiments were performed essentially as described (18). Briefly, 50 ng of human GDNF (PeproTech) was mixed with 250 ng of a fusion protein formed by rat GFRα1-Fc domain of human IgG (GFRα1-Fc, R&D Systems) in PBS. In some experiments, the related ligand NTN (human) and the Fc fusion of its cognate GFRα2 receptor (from rat) were also used. After 5 min of incubation at room temperature, the protein solution was added to the wells of an enzyme-linked immunosorbent assay plate (MaxiSorp, Nunc). The following day, the wells were rinsed by PBS and blocked with 2% skimmed milk powder in TBS (2% MTBS) for 1 h at room temperature. Equal amounts of RET<sub>EC</sub> mutants were added to the GDNF-GFRα1-Fc coated wells and to wells only coated with 2% MTBS. The binding was allowed to proceed for 1 h at room temperature. The washing was performed with TBS with 0.1% Tween 20 three times and TBS three times. Following washing, bound RET<sub>EC</sub> molecules were detected by a monoclonal anti-HA antibody at a 1:2000 dilution in 2% MTBS. Finally, the monoclonal anti-HA antibody was detected with an anti-mouse horseradish peroxidase-conjugated antibody (DAKO) at a 1:10000 dilution in MTBS. The reaction was developed by addition of 3,3’3,5-tetramethyl benzidine (TMB) substrate according to the instructions of the manufacturer (Pierce).

**Native Deglycosylation of Human RET<sub>EC</sub> and Binding Analysis**—To examine the influence of the N-linked carbohydrates attached to the ectodomain of RET, the carbohydrates were enzymatically removed under native conditions. Briefly, 1 μg of semipurified HA-tagged RET<sub>EC</sub> was deglycosylated by incubation with 20 units of PNGase F (Roche Applied Science) at 37 °C for 4 h. Half of the reaction was applied for binding experiments as described above. The remaining portion was taken for Western blot analysis to verify the removal of N-linked carbohydrates.
greater than 85% sequence identity. When compared with human RET<sup>EC</sup>, Xenopus RET<sup>EC</sup> showed negligible binding to the mammalian GDNF-GFRα1 complex in the concentration range tested (Fig. 1C), indicating that divergent regions between human and Xenopus RET<sup>EC</sup> may represent specific ligand binding determinants.

**A Strategy for Homologue-scanning Mutagenesis of the Human RET<sup>EC</sup>**—The inability of Xenopus RET<sup>EC</sup> to interact with the mammalian GDNF-GFRα1 complex despite its overall structural similarity to the human RET<sup>EC</sup> allowed us to use a homologue-scanning mutagenesis approach to study structure-function relationships in the RET<sup>EC</sup>. Based on the subdomain boundaries defined in a previous bioinformatics study on the RET<sup>EC</sup> (17), a series of chimeric molecules was constructed by swapping different subdomains between human and Xenopus RET<sup>EC</sup> (Fig. 2A). Stable CHO cell lines secreting different epitope-tagged chimeric Xenopus/human RET<sup>EC</sup> molecules were generated as described previously (18).

In a previous study, we found that certain subdomains of the RET<sup>EC</sup>, mainly CLD1, -2, and -3, have an intrinsic susceptibility to misfolding that makes them particularly vulnerable to inactivating mutations such as those found in patients with Hirschsprung disease (18). Misfolded RET<sup>EC</sup> molecules are retained intracellularly in the endoplasmic reticulum and eventually ubiquitinated and degraded, although a fraction may also get access to the extracellular space by direct leakage from the endoplasmic reticulum, particularly after overexpression (18). Folded and misfolded RET<sup>EC</sup> molecules can be distinguished by the sensitivity of the latter to Endo H (18). Upon exit from the endoplasmic reticulum, correctly folded glycoproteins lose sensitivity to Endo H as carbohydrates of higher complexity are added in the Golgi complex. The structural integrity of chimeric RET<sup>EC</sup> molecules was examined by subjecting the secreted proteins to Endo H treatment as described previously (18). As a control, RET<sup>EC</sup> proteins were treated with PNGase F, which removes sugars from both folded and misfolded proteins. As shown in Fig. 2A, all chimeric RET<sup>EC</sup> molecules were resistant to Endo H as expected (open arrowheads). These results indicated that this set of Xenopus/human chimeric RET<sup>EC</sup> molecules was folded correctly.
Distinct Clusters of Exposed Residues in CLD1 Are Required for Binding of Human RET ECD to the GDNF-GFRα1 Complex—Having established the existence of ligand binding determinants within CLD1, -2, and -3 of the human RET ECD, we set out to define more precisely the location and identity of functionally important residues in these three domains. The GETAREA 1.1 program (www.scsb.utmb.edu/getarea/area_man.html) was used to generate surface accessibility plots of CLD1, -2, and -3 of the human RET ECD using the coordinates of their modeled structures (17) (see the supplemental figure). Segments displaying more than 50% surface accessibility were examined for their degree of sequence similarity to analogous segments in the Xenopus RET ECD. Exposed segments of 6–12 residues in CLD1, -2, and -3 of the human RET ECD displaying more than 50% divergence from the equivalent Xenopus sequences were targeted for a second round of homologue-scanning mutagenesis (Fig. 3A). A total of 10 chimeric constructs (designated with the Roman numerals I–X) were generated and produced in the supernatants of stable CHO cell transfectants as above (Fig. 3B). With the exception of chimeric protein X, all other chimeras were resistant to Endo H digestion and therefore considered correctly folded (Fig. 3B). Equivalent amounts of wild type human RET ECD and chimeric proteins I–IX (Fig. 3C) were compared for their ability to bind the mammalian GDNF-GFRα1 complex in a solid-phase based binding assay as above.

Fig. 3. Distinct clusters of exposed residues in CLD1 are required for binding of human RET ECD to the GDNF-GFRα1 complex. A, alignment of the human and Xenopus RET ECD with conserved residues boxed in black, and surface-exposed, variable regions used to produce chimeric molecules boxed in gray. hRet, human RET; xRet, Xenopus RET. In B, chimeric RET ECD constructs produced in supernatants of stably transfected CHO cells grown at 30 °C were subjected to deglycosylation as indicated. RET ECD proteins were detected with an anti-HA antibody. Deglycosylation-resistant (solid arrowheads) and -sensitive (empty arrowheads) species are indicated. All constructs were sensitive to PNGase F digestion, as expected. Constructs I–IX were largely resistant to Endo H, whereas construct X was sensitive, indicating a misfolded protein. The diagram summarizes the chimeric molecules generated and their corresponding nomenclature. C, anti-HA tag blot of CHO cell supernatants showing that wild type hRET ECD and chimeric molecules I–IX were produced at comparable levels. IB, immunoblot. D, solid-phase binding assay of chimeric RET ECD molecules. Wells were coated with GDNF-GFRα1-Fc complex (solid bars) or PBS (white bars) and subsequently blocked with low-fat milk. Results were normalized to the binding of wild type human RET ECD. Control denotes supernatant from mock-transfected CHO cells. Shown are means ± S.D. of triplicate observations.

Distinct Clusters of Exposed Residues in CLD1 Are Required for Binding to the Mammalian GDNF-GFRα1 Ligand Complex—The ligand binding activity of chimeric RET ECD proteins was evaluated using a solid-phase binding assay with the GDNF-GFRα1 complex immobilized to 96-well plates. Chimeric molecules were also tested against a complex formed by human NTN and rat GFRα2. Equivalent levels of wild type and chimeric proteins were present in these experiments as demonstrated by Western blotting using an antibody against the HA epitope tag present in all the constructs (Fig. 2B). Xenopus RET ECD was not able to interact with the NTN-GFRα2 complex (data not shown). As shown in Fig. 2, C and D, binding activity to either ligand complex required all three N-terminal cadherin-like domains of RET ECD (i.e. CLD1, -2, and -3) to be of human origin. In contrast, the species of origin of CLD4 and the cysteine-rich domain had no impact on the ligand binding activity of chimeric RET ECD molecules (Fig. 2, C and D). Similar structure-activity profiles were observed toward both GDNF-GFRα1 and NTN-GFRα2 complexes, indicating that the RET ECD interacts with different members of the GDNF and GFRα families in a similar fashion. The fact that residues on both sides of the Ca2+ coordination site were required for ligand binding is in agreement with the recognized importance of Ca2+ for stabilizing the RET ECD in a conformation competent for ligand binding and receptor activation (Fig. 1B) (17, 22).
As shown in Fig. 3D, chimeras I, III, IV, V, and VI all displayed a reduction in binding as compared with the wild type human RETECD.

Chimeric protein I carries 4 amino acid replacements as compared with wild type human RETECD, namely S32L, D34K, A35D, and W37Y, suggesting that one or more of those positions are crucial for the interaction of the RETECD with the GDNF-GFRα1 complex. Based on the modeled structure of CLD1 (17), residues in region III are predicted to form part of a loop between the C and D β-strands of this domain. This exposed segment is shorter and highly divergent in the Xenopus RETECD, indicating that residues in this loop region may also contribute to the binding of the human RETECD to the GDNF-GFRα1 complex.

In contrast to chimeras I, III, and V, chimeric protein IV retained ~20% binding to the GDNF-GFRα1 complex (Fig. 3D). Of the 8 amino acid exchanges in this region, three involve the replacement of positively charged residues by uncharged residues (Fig. 3A), indicating that one or more of those charges are important for formation of the GDNF-GFRα1-RET complex. The complete absence of detectable binding in chimeric protein V indicates that one or more of the residues exchanged in this region are important for ligand binding. Of the 9 amino acid differences between the human and Xenopus sequences in this region, R133L and W139N may be the most significant ones as they involve residues that are enriched in known protein-protein interfaces (25). Finally, a 60% reduction in ligand binding was observed in chimeric protein VI (Fig. 3D), which involves 9 amino acid replacements in CLD2 (Fig. 3A). Of note, this was the only set of mutations outside CLD1 that affected the interaction of the RETECD with the GDNF-GFRα1 complex. Interestingly, three of the exchanges in this region involve the replacement of two polar and one uncharged residue by positively charged residues (Fig. 3A). Taken together, they indicate that although CLD1, -2, and -3 of human RETECD are all required for ligand binding, the most important determinants appear to be concentrated in CLD1, the most N-terminal subdomain of the human RETECD.

**N-linked carbohydrates in human RETECD are dispensable for ligand binding**—Protein glycosylation can have a modulating effect on protein-protein interactions, and introduction of N-linked glycosylation sites has been used as a mutagenesis strategy (26). The human RETECD is abundantly N-glycosylated, but the role of this post-translational modification in ligand binding is unknown. Interestingly, predicted N-glycosylation sites in the human RETECD are not evenly distributed, but the majority (9 of 12) of them appear downstream of the Ca²⁺ coordination site, in accord with the location of ligand binding determinants in the CLD1. We treated the human RETECD with PNGase F under native conditions and examined its ability to bind to the GDNF-GFRα1 complex. Treatment with PNGase F resulted in complete deglycosylation of the native protein, comparable with that obtained after prior denaturation (Fig. 4A). As shown in Fig. 4B, RETECD incubated in deglycosylation buffer with or without PNGase F without prior denaturation showed no loss of binding as compared with a non-treated control. Denaturation prior to enzymatic deglycosylation resulted in complete loss of binding, as expected (Fig. 4B). Thus, N-linked carbohydrates are unlikely to play a role in the assembly of the RET-GDNF-GFRα1 complex and may instead be of importance for the folding and maturation of the RETECD in the secretory pathway, as suggested previously (18).

**DISCUSSION**

The receptor tyrosine kinase RET has remained in the limelight ever since its discovery as a transforming protein in 1987 (27) and the subsequent elucidation of its participation in the receptor complex for GDNF family ligands (10, 28, 29). Despite having an extracellular domain of more than 600 residues, RET cannot engage any of these ligands directly but requires the auxiliary GFRα receptors for activation (29, 30). Although the RETECD was known to make direct contacts with both GDNF ligands and GFRα receptors in the complex, binding determinants in the RETECD molecule and the overall architecture of the complex remained to be characterized.

In this study, we set out to identify functional determinants in the RETECD responsible for its association with the GDNF-GFRα1 complex using a homologue-scanning mutagenesis approach based on the differential abilities of human and Xenopus RETECD to interact with ligand complexes of mammalian origin. Using this approach, binding determinants were found to be concentrated in the N-terminal CLD1 of the human RETECD. Within this region, three discrete segments, ranging from 6 to 12 residues (i.e. I, III, and V in Fig. 3A), could not be replaced by equivalent sequences from Xenopus RETECD without complete loss of activity, indicating that these segments are required for the binding of the human RETECD to the mammalian GDNF-GFRα1 complex. Importantly, these replacements had no detectable effects on protein production, stability, secretion, or folding, at least at 30 °C. Because of their relatively high solvent accessibility, these epitopes are likely to be directly involved in the interaction of RET with its ligands. When visualized on the modeled three-dimensional structure of the CLD1 (17), regions I, III, and V are all localized on the same face of the model (Fig. 5A), delineating a probable surface for interaction with the GDNF-GFRα1 complex. In support of this notion, the only site of N-glycosylation known in the human CLD1, namely Asn-98, is located in the opposite side of the

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S Kjær, unpublished observations.
domain, a position that is sterically compatible with the proposed location of the ligand binding interface.

Bioinformatics analysis of the RETECD has recently indicated a structural organization resembling that of classical cadherins with four cadherin-like domains followed by a C-terminal cysteine-rich domain not related to cadherin sequences (17). The X-ray crystal structure of the complete extracellular domain of C-cadherin has recently been solved, revealing an elongated rod-shaped structure (24). Two types of interactions between different cadherin molecules could be defined in these crystals: cis interactions were formed laterally between adjacent cadherin molecules, whereas trans interactions linked cadherin molecules in opposite orientations, presumably representing the kind of interactions responsible for cell-cell contact. The trans interface was defined by a conserved tryptophan side chain (Trp-2) at the N-terminal end of the cadherin molecule from one cell, which was shown to insert into a hydrophobic pocket in the cadherin molecule from the opposing cell (24). The importance of Trp-2 for cadherin-mediated cell adhesion had been inferred independently from structure-function analyses (31). However, Trp-2 is not conserved in RETECD sequences from different organisms, and no functionally analogous residues can be identified in the modeled structure of the RETECD (17). Moreover, no adhesive function has to date been attributed to RET molecules (32).

Given the pivotal role played by the CLD1 in the interaction of RET with its ligands, a straight rod-like organization similar to that of classical cadherins would place the major ligand binding site in the RETECD away from the plasma membrane, where the membrane-anchored GDNF-GFRα1 complex is likely to be. In fact, structure-function studies of the GFRα1 molecule have indicated that the N-terminal domain of this receptor is dispensable for ligand binding and have instead localized the binding determinants toward the middle and C-terminal portions of the molecule (12), in agreement with a membrane-proximal site of complex assembly. How could these apparently contradictory observations be reconciled?

An analogous topological problem has been posed by the cytokine receptor complex formed by gp130, IL-6Rα, and IL-6. Like RET, gp130 has a large multidomain extracellular region, and ligand binding determinants have been localized to the three most N-terminal domains (33). On the other hand, IL-6Rα, like GFRα1, has ligand binding determinants in its membrane-proximal domains (34). A recent crystallographic analysis of the assembly of this complex revealed a bent structure for gp130 and IL-6R with their ligand binding domains forming a “table” that rests on “legs” composed by the more C-terminal domains of the molecules (35). In an analogous fashion, we hypothesize that a bent arrangement of the RETECD would allow its ligand binding domain to reach the GDNF-GFRα1 complex located closer to the membrane (Fig. 5B). It is worth noting that this arrangement would not be possible had the RETECD retained all the interdomain Ca$^{2+}$-binding sites that characterize classical cadherins. As mentioned previously, the single Ca$^{2+}$-binding site in the RETECD is located at the interface of CLD2 and CLD3, suggesting a straight and rigid conformation for these two domains. On the other hand, the lack of Ca$^{2+}$-binding sites in all of the other interdomain regions suggest a greater degree of flexibility as compared with classical cadherins that may allow the RETECD to bend toward the cell membrane. Clearly, validation of this hypothesis awaits the structural determination of the RETECD in complex with GFRα1 and GDNF.

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