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Direct interactions among Ret, GDNF and GFRα1 molecules reveal new insights into the assembly of a functional three-protein complex

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Abstract

The glial-cell-line-derived neurotrophic factor (GDNF) ligand activates the Ret receptor through the assembly of a multiprotein complex, including the GDNF family receptor α1 (GFRα1) molecule. Given the neuroprotective role of GDNF, there is an obvious need to precisely identify the structural regions engaged in direct interactions between the three molecules. Here, we combined a functional approach for Ret activity (in PC12 cells) to cross-linking experiments followed by MS-MALDI to study the interactions among the purified extracellular region of the human Ret, GDNF and GFRα1 molecules. This procedure allowed us to identify distinct regions of Ret that are physically engaged in the interaction with GDNF and GFRα1. The lack of these regions in a recombinant Ret form results in the failure of both structural and functional binding of Ret to GFRα1/GDNF complex. Furthermore, a model for the assembly of a transducing-competent Ret complex is suggested.

Keywords: Tyrosine kinase receptor; MALDI mass spectrometry; GDNF; Ret

1. Introduction

The *ret* protooncogene encodes a tyrosine kinase receptor that plays a crucial role in kidney morphogenesis and in the survival and differentiation of several subpopulations of neurons in the peripheral and central nervous systems [1]. The Ret protein consists of three functional regions: the extracellular region, including four N-terminal cadherin-like domains (named CLD1 to CLD4) followed by a single cysteine-rich domain (CRD), the transmembrane region and the intracellular region formed by a bipartite tyrosine kinase domain [2].

Four members of the glial cell line-derived neurotrophic factor (GDNF) family, including GDNF, neurturin, artemin and persephin represent the Ret ligands. Ret activation by these neurotrophic factors is mediated by their binding to one of the four different glycosyl phosphatidylinositol-anchored receptors, termed GDNF family receptor α (GFRα) 1 to 4 [3]. GDNF has potent trophic effects on dopaminergic nigral neurons, indicating this factor as a promising protective agent in neurodegenerative diseases. In various animal models of Parkinson's disease, GDNF has been shown to prevent the neurotoxin-induced death

Abbreviations: CLD, cadherin-like domain; CRD, cysteine-rich domain; GDNF, glial cell line-derived neurotrophic factor; GFRα1, GDNF family receptor α1; EC-Ret^{wt}, Ret extracellular portion; EC-Ret$^{1-387}$, Ret extracellular portion deleted of CLD4 and CRD.

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of dopamine neurons and can promote functional recovery (for a review, see Ref. [4]). Despite a cross-talk between the different ligands–GFRαs pairs, a preferred coreceptor molecule exists for each ligand, GDNF being the preferred high-affinity ligand for GFRα1. On the other hand, in the absence of GFRα1, Ret is not able to bind GDNF, whereas it can interact weakly with GFRα1 even in the absence of GDNF [5–7]. GDNF-dependent activation of Ret implicates its recruitment to the GFRα1/GDNF complex, leading to Ret dimerisation and autophosphorylation at specific cytoplasmic tyrosine residues [1]. Several structural elements in both GDNF and GFRα1 that are required for the formation of the complex have been tentatively defined [8–10]. By constructing a set of chimeric and truncated coreceptors and analysing their ligand binding and signaling capabilities, a central domain in the GFRα1 molecule was identified as necessary for physical and biochemical interaction with both GDNF and Ret molecules [11]. The crystal structure of the second half of this central domain has been recently solved, revealing it as an independent folding unit [12]. In addition, homologue-scanning mutagenesis of GDNF allowed identifying residues located along the second finger of the ligand according to the crystal structure as critical for the interaction with GFRαs molecules [8,9]. On the other hand, by constructing chimeric molecules constituted by the human and the *Xenopus* regions of the extracellular domain of Ret fused together, the binding determinants for GFRα1/GDNF complex have been found to be mainly concentrated in the first CLD1 of Ret [13]. However, the identification of regions and specific residues directly engaged in the intermolecular interactions among the Ret, GDNF and GFRα1 molecules and needed to form a functionally active transducing complex is still questioned.

This study is intended to identify key amino acids engaged in direct interactions between the three molecules. Nowadays, cross-linking reagents are widely used in the assessment of contact regions in protein–protein interaction mainly in combination with mass spectrometry as the analytical methodology able to precisely identify covalently linked residues [14–17].

Therefore, we used a proteomic-based approach to study the interactions among the pure extracellular region of human Ret, the GDNF and the GFRα1 molecules. The three proteins were incubated in vitro and treated with a limited excess of cross-linking reagents. The reaction products were fractionated on SDS polyacrylamide gel (SDS-PAGE), and the individual cross-linked species were characterised by mass spectrometry fingerprinting analysis. The obtained results led to the identification of distinct regions in the extracellular region of Ret that are required for the assembly of a transducing-competent three-component complex. Signal transduction experiments in PC12 cells fully confirmed the structural analyses findings of the used approach.

2. Materials and methods

2.1. Cloning, expression and purification of EC-Ret proteins

The entire Ret receptor extracellular portion (EC-Ret^{wt}) was produced as previously reported [18]. The protein containing the first three N-terminal CLDs of EC-Ret^{wt} but lacking CLD4 and CRD (named EC-Ret$^{1–387}$) was produced as follows.

A fragment encoding residues 1–387 of EC-Ret^{wt} was amplified using Taq polymerase in standard PCR conditions (F:5′AGTGTTTAAATTTAAGCTTGCGGCCG3′ and R:5′CACATTCAAATGTAGTAAGGATCCGC3′). The C-terminus of the resulting PCR fragment was ligated to a fragment containing a tobacco etch virus (TEV) protease cleavage site followed by the two IgG binding domains of *S. aureus* protein A into a pcDNA 3.1 (+) expression vector (Invitrogen). The resulting plasmid was transiently transfected in HEK 293T cells at 90% confluence plated on to six 15-cm plates using the Lipofectamine 2000 reagent (Invitrogen). The ultrafiltrated culture medium (15 ml final volume) was loaded onto an IgG Sepharose 6 Fast Flow column (Amersham-Pharmacia, 0.5×3 cm), and the sample was eluted in the presence of TEV protease. The recovered sample was fractionated onto a Superdex 200 High Load column (Amersham-Pharmacia, 1×30 cm) connected to a Fast Protein Liquid Chromatography (FPLC) system. The total protein yield was approximately 500 ng/ml of culture medium.

2.2. Cell culture, preparation of cell extracts and immunoblotting analysis

PC12/wt (PC12 cells stably transfected with human Ret^{wt} receptor) and PC12-α1/wt (PC12 cells stably transfected with both human Ret^{wt} receptor and GFRα1 coreceptor) cells were grown as previously reported [19]. When indicated, GDNF (Promega) or recombinant rat GFRα1/-Fc chimera (R&D System) were added to the culture medium.

Cells were washed twice in ice-cold PBS then lysed in 50 mM Tris–HCl, pH 8.0 buffer containing 150 mM NaCl, 1% Nonidet P-40, 2 μg/ml aprotinin, 1 μg/ml pepstatin, 2 μg/ml leupeptin, 1 mM Na_3VO_4. The solution was centrifuged at 16,000 *g* for 30 min at 4 °C, and the residue was discarded. Protein concentration was determined by the Bradford assay using bovine serum albumin as standard. After SDS-PAGE, proteins were electroblotted to polyvinylidene difluoride membranes (Millipore) and detected with the indicated primary antibodies and peroxidase-conjugated secondary antibodies using the enhanced chemiluminescence system (Amersham-Pharmacia). The primary antibodies used were the following: anti-Ret (H-300) and anti-ERK1 (C-16; Santa Cruz Biotechnology), anti-phosphoRet (Cell Signaling), anti-phospho44/42 MAP Kinase monoclonal antibodies (E10; Cell Signaling). When indi-

cated, membranes were stripped in 62.5 mM Tris–HCl pH 6.7, 0.1 M 2-mercaptoethanol, 2% SDS, for 30 min at 55 °C. The immunoblots shown are examples of at least three independent experiments.

2.3. Neurite outgrowth bioassay

PC12-α1/wt cells were plated at equal density on 12-well culture plate. To evaluate the effects of EC-Ret proteins on cell differentiation, cells were incubated with 50 ng/ml GDNF together with the appropriate protein at a final concentration of 10 μg/ml. At least 15 random fields were photographed 24 h after GDNF stimulation by using a phase-contrast light microscope, and 30 cells per frame were counted and scored as having neurites or not. A neurite was operationally defined as a process outgrowth that was long more than twice the diameter of cell body.

2.4. Deglycosylation procedure

Treatment with peptide N-glycosidase F (PNGase F) or endoglycosidase H (Endo H) was performed according to the protocol supplied by the manufacturer (New England Biolabs).

2.5. Chemical modifications

Cross-linking reactions were carried out in a total volume of 80 μl of 10 mM sodium phosphate buffer, pH 7.5. Recombinant GDNF, GFRα1 and EC-Ret^{wt} or EC-Ret$^{1–387}$ were mixed in equimolar amount (2×10^{-6} mol/l) and incubated at 25 °C for 30 min. Following complex formation, either suberic acid bis (3-sulpho-*N*-idroxysuccinimide ester) sodium salt (BS3) or 1-ethyl-3-(3-dimethylamino propyl)-carbodiimide (EDAC) cross-linking reagents (Sigma) were added to the mixture. Several preliminary experiments were carried out to precisely determine the optimal excess of chemical reagents. A molar excess of 1:2500 (mol/mol) for both reagents was eventually defined, and the modification reactions were allowed to proceed at 25 °C for 3 h.

2.6. SDS-PAGE analysis and "*in situ*" digestion

Cross-linking reactions were terminated by quenching the excess of reagents with 20 μl of gel loading buffer (Tris–HCl 180 mM, 6% SDS, 30% Glycerol, Blu Bromophenol 0.03%, pH 6.8). Samples were heated at 100 °C for 5 min and then separated by gel electrophoresis through a 7% tricine SDS polyacrylamide gel. Proteins were detected by silver staining, and positively stained protein bands were excised from the gel and washed in deionised MilliQ grade water (2 times, 10 min). The excised spots were destained with 100 μl of destaining solution (30 mM $K_3Fe(CN)_6$, 100 mM $Na_2S_2O_3$) and washed with 100 μl of 100 mM ammonium bicarbonate buffer. After 5 min, an equal amount of acetonitrile was added. Protein samples were reduced by incubation in 10 mM dithiothreitol (DTT) for 45 min at 56 °C. Reducing solution was removed by acetonitrile wash, and free cysteines were alkylated by treatment with 55 mM iodoacetamide for 30 min at room temperature in the dark. The supernatant of the alkylating solution was discarded, and the reaction was stopped by washing gel pieces with ammonium bicarbonate buffer and acetonitrile. Enzymatic digestion was carried out by incubating gel pieces with 30 μl of a 1 ng/μl trypsin solution in 10 mM ammonium bicarbonate at 4 °C for 2 h. The excess of trypsin solution was then removed, and a new aliquot of 10 mM ammonium bicarbonate was added; samples were incubated for 18 h at 37 °C. A minimum reaction volume was used to obtain the complete hydration of the gel. Peptides were then extracted by washing the gel particles with 0.1% TFA in 50% acetonitrile at room temperature. Peptides were purified by using ZipTip Pipette Tips following manufacturer protocol (Millipore Billerica, USA).

2.7. MALDI-MS analyses

MALDI-TOF mass spectra were recorded using an Applied Biosystem Voyager DE-PRO instrument. A 1-μl aliquot of a mixture of analyte and matrix solution (alfa-cyano-hydroxycinnamic acid 10 mg/ml in 66% ACN, 0.1% TFA, in MilliQ water) was applied to the metallic sample plate and dried down at room temperature. Mass calibration was performed using external peptide standards. Raw data were analysed using the computer software provided by the manufacturer and reported as monoisotopic masses. The accuracy of the mass determinations was always within 30 ppm. The percentage of noninterpreted signals was less than 10%.

3. Results

3.1. Production of EC-Ret^{wt} and EC-Ret$^{1–387}$

To perform cross-linking experiments, we took advantage of pure protein preparations corresponding to the entire Ret extracellular region, protein ending at amino acid 636 (EC-Ret^{wt}) [18], and of a deletion mutant encompassing CLD1, two and three of the native protein but lacking CLD4 and CRD (EC-Ret$^{1–387}$). The recombinant Ret truncated form was produced as described in Materials and methods section and characterised for its homogeneity. An aliquot of 30 μg of purified EC-Ret$^{1–387}$ loaded onto a Superdex 200 (FPLC System) gave rise to a symmetric chromatographic peak with an apparent molecular mass of approximately 80 kDa, which was compatible with a homogeneous preparation of the protein in a correctly folded conformation. However, the correct folding of EC-Ret$^{1–387}$ was further tested by

endoglycosidases assays. As recently reported, N-linked glycosylation of the extracellular domain of human Ret is not required for ligand binding, but it is relevant for the folding and the maturation of the protein [20,21]. We analysed the sensitivity of both EC-Ret^{wt} and EC-Ret$^{1–387}$ proteins to PNGase F and Endo H. It is well known in fact that PNGase F removes the oligosaccharide chains from all glycoproteins regardless of their folding state, whereas Endo H deglycosilates misfolded proteins that are retained in ER. EC-Ret$^{1–387}$ was incubated with either PNGase F or Endo H, and the reaction products were analysed by SDS-PAGE (Fig. 1). The same experiments were also carried out on native Ret for comparison. As shown in Fig. 1, the correct folding and maturation state of purified EC-Ret$^{1–387}$ were confirmed by its sensitivity to PNGase F and resistance to Endo H treatment. Comparable results were obtained with the EC-Ret^{wt} preparation (Fig. 1).

3.2. CLD4 and CRD are required for binding of EC-Ret^{wt} to GFRα1/GDNF

By using the EC-Ret^{wt} and the deletion mutant EC-Ret$^{1–387}$ proteins, we determined whether the extracellular domain of Ret retains in vitro the ability to form a multiprotein complex with GDNF and GFRα1 molecules. Equimolar amounts of recombinant GDNF, GFRα1 and EC-Ret^{wt} (2 μM final concentration each) were incubated in vitro in phosphate buffer and allowed to interact to obtain complex formation. In agreement with the methods reported in literature for the in vitro chemical cross-linking (see Ref. [14] as representative reference), low concentrations of the three protein components were chosen to avoid unspecific protein–protein interactions. Moreover, preliminary experiments were carried out to precisely determine the appropriate molar excess of reagents to limit the amount of cross-linking bonds and to avoid an excess of undesired surface labelling. As previously reported, in fact, a large number of chemical cross-links might perturb the native conformation of the complex, whereas modification of surface amino acids might greatly complicate the interpretation of the mass spectral data [15,16].

The cross-linking agent BS3, a bifunctional reagent linking amines to amines, thus selective for the N-terminal amino group and lysine side chains, with a spacer arm of 11.4 Å [22], was then added to the complex. The reaction was stopped by direct dilution with sample buffer, and the modified samples were fractionated by SDS-PAGE as shown in Fig. 2 (left panel, lanes 3–6). Isolated GFRα1 and EC-Ret^{wt} proteins were also loaded onto the gel as specific electrophoresis mobility standards (lanes 2 and 7). Similar experiments were carried out using the truncated form of Ret, EC-Ret$^{1–387}$ (lanes 8–10).

Fig. 2 shows that the cross-linking reactions resulted in the production of covalently linked species that were named 1A, 1B, 1C and 1D according to their electrophoretic mobility (lanes 3–6). When the truncated EC-Ret$^{1–387}$ was used in the experiments, only three protein bands, namely, 2A, 2B and 2C, were observed (lanes 8 and 9). No evidence of a high molecular weight band corresponding to the 1D species was observed in these samples.

The protein bands were excised from the gel, reduced, alkylated and in situ digested with trypsin [23]. The resulting peptide mixtures were analysed by MALDI-MS following the mass fingerprinting procedure [15]. As an example, Fig. 3 shows the partial MALDI mass spectrum of the peptide mixture from band 1D, the specific complex only formed in the presence of native Ret protein. The mass spectral analysis revealed the occurrence of mass signals corresponding to linear peptides originated from the GDNF, GFRα1 and Ret sequences, thus indicating that this band corresponds to a three-component covalent complex involving GDNF, GFRα1 and EC-Ret^{wt}. Moreover, a series of signals were identified in the mass spectra that could not be assigned to any linear peptide. These signals were not detected in the mass mapping of isolated GDNF, GFRα1 and Ret carried out as reference and were then interpreted as modified fragments. These modifications might have been generated either by surface labelling, i.e., BS3 modification of exposed residues or by intra- and/or intermolecular cross-links. The assignments of these mass signals were then performed on the basis of the mass values recorded in the spectra and the specificity of the trypsin digest [24], taking

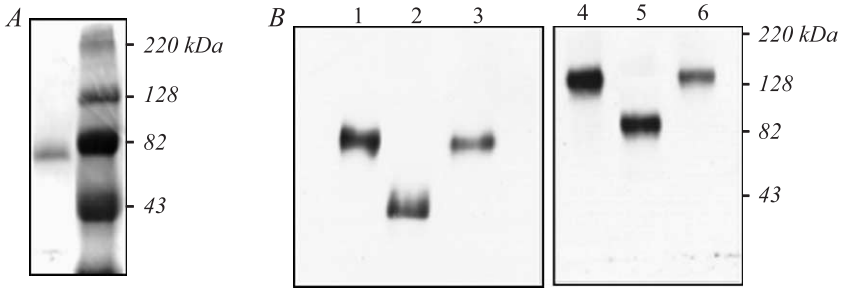

Fig. 1. Deglycosylation analysis of EC-Ret proteins. (A) SDS-PAGE (10% acrylamide) of a pure sample of EC-Ret$^{1–387}$ revealed by Coomassie Brilliant Blue staining; molecular-mass standards are shown. (B) EC-Ret$^{1–387}$ or EC-Ret^{wt} before (lane 1 or 4, respectively) and following treatment with PNGase F (lane 2 or 5, respectively) or Endo H (lane 3 or 6, respectively) immunoblotted with anti-Ret antibodies.

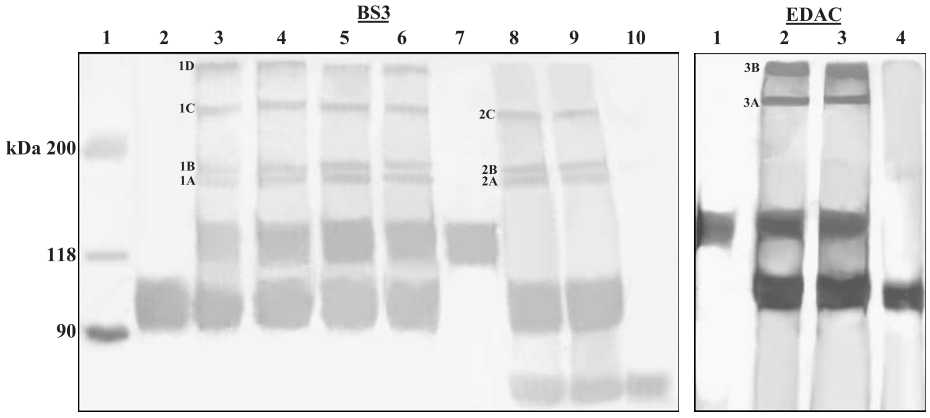

Fig. 2. Silver-stained SDS PAGE analysis of samples from BS3 and EDAC cross-linking experiments. The samples loaded on the gel are as follows: BS3 (left panel), markers (lane 1); GFRα1 (lane 2); cross-linking reaction between EC-Ret^{wt}, GDNF and GFRα1 (lanes 3–6, shown in triplicate); EC-Ret^{wt} (lane 7); cross-linking reaction between the truncated EC-Ret$^{1–387}$, GDNF and GFRα1 (lanes 8–9, shown in duplicate); EC-Ret$^{1–387}$ (lane 10). EDAC (right panel), EC-Ret^{wt} (lane 1); cross-linking reaction between EC-Ret^{wt}, GDNF and GFRα1 (lanes 2–3, shown in duplicate); GFRα1 (lane 4).

into account all the possible modifications occurring in the peptide. The mass searching of the modified peptides was carried out with a mass tolerance of 30 ppm, thus leading to univocal attributions of the signals, as summarised in Table 1. As an example, the signal at *m*/*z* 1045.6 was assigned to the GFRα1 peptide 89–95 carrying a covalently linked BS3 moiety, while the signal at *m*/*z* 1320.6 revealed the occurrence of an intramolecular cross-link involving the fragments 89–91 and 92–97 of the GFRα1 sequence. Following this procedure, a number of real intramolecular covalent bonds linking the three proteins were detected. The signal at *m*/*z* 1475.8 was assigned to the covalent cross-link between the peptide 91–97 of GFRα1, oxidized at level of Met93, with the GDNF 1–4

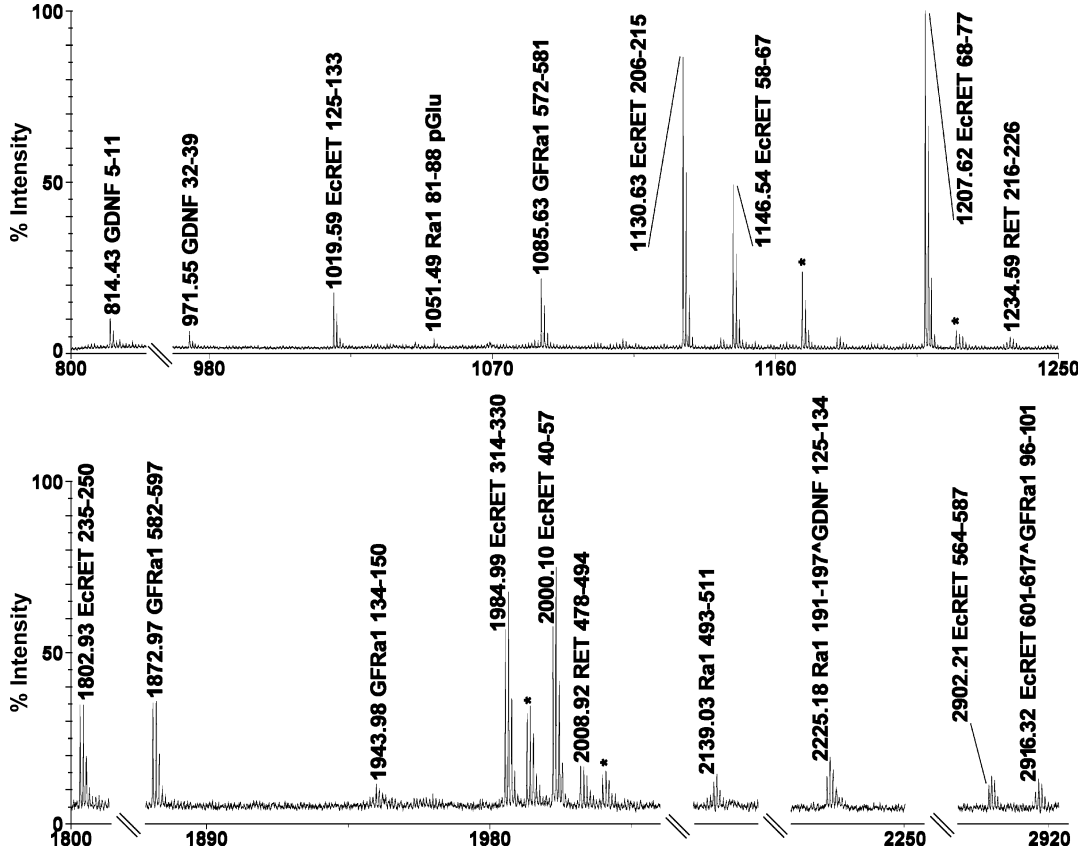

Fig. 3. Partial MALDI mass spectrum of the peptide digest from band 1D. Mass signals were assigned to the corresponding peptides within GDNF, EC-Ret^{wt} and GFRα1 sequences on the basis of their mass values. Tripsin autoproteolysis peaks are marked with an asterisk.

Table 1
Mass values and corresponding fragments from the BS^3 cross-linking experiment

Measured mass (*m*/*z*)	Modified peptides	Residues	Theoretical mass (*m*/*z*)	Found in band
1045.6	Rα1 89–95^BS3 (internal)	Rα1 90 Rα1 94	1045.6	1D
1079.5	Rα1 89–91^Rα1 92–95(ox)	Rα1 90 Rα1 94(95)	1079.5	1D
1320.6	Rα1 89–91^Rα1 92–97(ox)	Rα1 90 Rα1 94(95)	1320.6	1A, 1B, 1C, 1D, 2A, 2B, 2C
1429.6	Rα1 92–97(ox)^Rα1 191–194	Rα1 94(95) Rα1 191	1429.6	1A, 1B, 1C, 1D, 2B
1446.8	Rα1 91–97(ox)^GDNF 28–31	Rα1 94(95) GDNF 29	1446.84	1D
1461.8	Rα1 89–95^GDNF 28–31	Rα1 94(90,95) GDNF 29	1461.8	1D
1475.8	Rα1 91–97(ox)^GDNF 1–4	Rα1 94(95) GDNF 1	1475.8	1D
1497.8	GDNF 1–4^GDNF 28–35	GDNF 1 GDNF 29	1497.8	1D
1699.8	Rα1 95–97^Rα1 39–47	Rα1 95 Rα1 45	1699.8	1D
2225.2	Rα1 191–197^GDNF 125–134	Rα1 191 GDNF 129	2225.1	1A, 1B, 1D, 2B, 2C
2916.3	Rα1 96–101^RET 601–617	Rα1 97 RET 603	2916.3	1D

fragment. Moreover, the peak at *m*/*z* 2225.2 was identified as originated by the peptide 191–197 of GFRα1 covalently linked to the fragment 125–134 of GDNF, confirming that coreceptor/ligand interaction occurred through these regions of the two proteins. As a whole, a detailed examination of the data demonstrated that the GFRα1 region 91–97 is in close contact with the N-terminal portion of the GDNF molecule (region 1–31) via covalent cross-links involving either Lys94 or Lys95 of GFRα1 and the N-terminal amino group and Lys29 of GDNF. A further cross-link between the coreceptor and the ligand was established through Lys129 in the C-terminal portion of GDNF and either Lys191 or 194 of GFRα1, as reported in Table 1. Finally, a direct interaction between GFRα1 and EC-Ret^{wt} was established through a chemical linkage joining Lys97 of the coreceptor and Lys603 of Ret, as indicated by the signal at *m*/*z* 2916.3 involving the GFRα1 96–101 peptide and the EC-Ret^{wt} 601–617 fragment. It should be emphasised that no direct chemical cross-links between EC-Ret^{wt} and the GDNF ligand could be identified in the analysis.

The MALDI mass spectral analyses of the peptide mixtures from bands 1A, 1B and 1C occurring in the presence of the native EC-Ret^{wt} form showed the presence of signals corresponding to peptides generated from tryptic hydrolysis of the GDNF and GFRα1 molecules. No mass signals corresponding to Ret sequences were detected, indicating that these bands corresponded to heterodimeric complexes consisting of GDNF and GFRα1 proteins. The occurrence of three bands consisting of the same two proteins but showing different electrophoretic mobilities was interpreted as due to the different stoichiometry of the cross-linked complexes between GFRα1 and the GDNF ligand. On the basis of their electrophoretic mobility referred to a calibration curve, the band 1C was tentatively attributed to a 2:2 GDNF/GFRα1 complex, whereas bands 1A and 1B were assigned to GDNF/GFRα1 complex with one GFRα1 molecule and different GDNF composition.

As reported in Table 1, several mass signals were detected in the spectra that corresponded to intermolecular covalently linked peptides, confirming that the region 191–197 of GFRα1 is directly joined to the C-terminal domain of GDNF. Interestingly, no linkages involving the N-terminal region of GDNF were detected.

Similar results were obtained in the analysis of the 2A, 2B and 2C bands that essentially confirmed the structural data obtained on the interaction between the ligand and the receptor occurring in the presence of the truncated EC-$Ret^{1–387}$.

The possible role of ionic interactions in the formation of the complex was investigated by selecting the acidic residues of the proteins for cross-linking formation. Following incubation of equimolar amounts of GDNF, GFRα1 and EC-Ret^{wt}, the protein carboxyl groups were activated by EDAC and allowed to react with amino groups to form zero-length intermolecular bonds [25]. The reaction mixture was then fractionated by SDS-PAGE (Fig. 2, right panel), and the protein bands were digested in situ. The triptic mixture from 3A and 3B bands were analysed by MALDI-MS following the procedure as described, revealing the presence of linear peptides generated from enzymatic digestion of the GDNF, GFRα1 and Ret proteins, thus indicating the presence of a ternary complex. All the mass signals occurring in the spectra were attributed as described above. The analysis of the mass signals corresponding to cross-linked peptides essentially confirmed previous cross-linking

Table 2
Mass values and corresponding fragments from the EDAC cross-linking experiment

Measured mass (*m*/*z*)	Cross-linked peptides	Cross-linked residues	Theoretical mass (*m*/*z*)
1872.0	GFRα1 91–95(ox)^GDNF 5–14	GFRα1 94(95) GDNF 13	1872.0
2726.2	GFRα1 92–97(ox)^EC-Ret^{wt} 478–494	GFRα1 94(95) EC-Ret^{wt} 480	2726.3
3301.7	GFRα1 91–101(ox)^GDNF 13–29	GFRα1 94(95) GDNF 24	3301.7

pattern, as illustrated in Table 2. The GDNF N-terminal portion is in close contact with the 91–97 region of the receptor GFRα1, as demonstrated by the cross-links between Glu13 and Glu24 of GDNF with either Lys94 or 95 from GFRα1. Moreover, a covalent linkage joining the 92–97 region of GFRα1 and Glu480 of Ret was identified, thus further supporting the occurrence of a direct interaction between EC-Ret^{wt} and the coreceptor. The mass fingerprinting analyses of the cross-linked bands corresponding to the GDNF/GFRα1 complex essentially confirmed the results obtained with BS3.

3.3. EC-Ret$^{1–387}$ does not compete with membrane-bound Ret^{wt} for binding to GDNF

To further confirm that the CLD4 and CRD are required for binding to GFRα1/GDNF complex, we investigated whether EC-Ret$^{1–387}$ may act as a competitive inhibitor for membrane-bound Ret. Indeed, as previously reported [18], EC-Ret^{wt} inhibits the GDNF-induced Ret activity by competing the membrane-bound Ret receptor for ligand binding. We determined whether EC-Ret$^{1–387}$ could interfere with the GDNF-induced stimulation of Ret. We took advantage of a PC12 derivative cell line expressing human Ret^{wt} (PC12/wt). As shown in Fig. 4A, in the presence of soluble GFRα1, GDNF stimulates Ret tyrosine phosphorylation (compare lane 2 to lane 1). Treating cells with increasing amounts of EC-Ret$^{1–387}$ did not affect the Ret phosphorylation (compare lanes 3 and 4 to lane 2), whereas EC-Ret^{wt}, used as a positive control, greatly decreased Ret tyrosine phosphorylation level, almost reaching the basal values (compare lanes 5 and 6 to lane 2). Accordingly, phosphorylation of the Ret downstream effector, ERK, was undetectable in the absence of the ligand (Fig. 4B, lane 1) and strongly stimulated by GDNF in the presence of soluble GFRα1 (lane 2), thus suggesting the integrity of the signal transmission pathway. The truncated EC-Ret$^{1–387}$ was not able to inhibit the GDNF-induced ERK phosphorylation (lanes 3 and 4), whereas, as expected, EC-Ret^{wt} behaved as a potent inhibitor (lanes 5 and 6).

As an appropriate control, we verified that the 10-min treatment of both EC-Ret^{wt} and EC-Ret$^{1–387}$ at 37 °C in RPMI medium at a final concentration of 2 μg/ml did not cause protein aggregation or misfolding of the proteins. The elution profile from a Superdex 200 column of the treated proteins (Fig 4C) gave rise to a symmetric chromatographic peak, with an apparent molecular mass compatible with the size of 120 and 80 kDa for EC-Ret^{wt} and EC-Ret$^{1–387}$, respectively, thus indicating the correct conformation state of the molecules. The same results were obtained at a final concentration of 4 μg of protein/ml of culture medium (not shown).

These findings indicate that the presence of CLD4 and CRD in the extracellular region are needed to compete Ret receptor for binding to GDNF.

We thus determined the biological effects of both EC-Ret^{wt} and EC-Ret$^{1–387}$ proteins on Ret-dependent cell

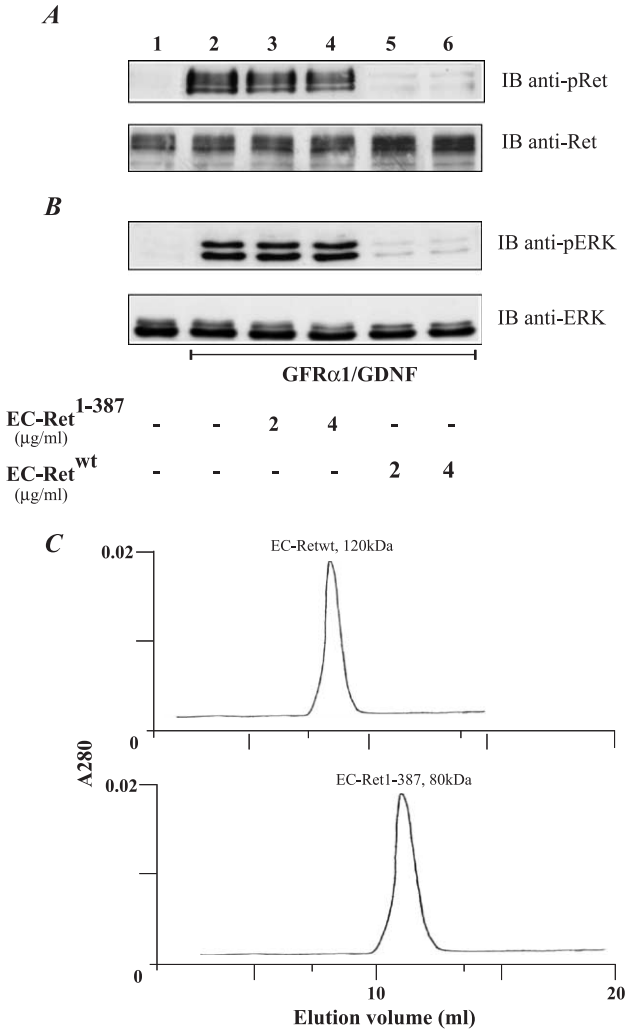

Fig. 4. Effect of EC-Ret proteins on Ret-mediated signaling. (A) PC12/wt cells (350,000 cells/6 cm plate) were starved for 4 h and then treated for 10 min with preincubated mixtures (5 ml final volume of RPMI medium), including GDNF (1.6 nM), soluble GFRα1 (1.6 nM) and EC-Ret$^{1–387}$ or EC-Ret^{wt} at the indicated concentrations. Cell lysates were immunoblotted with anti-phosphoRet antibodies (upper panel), the same filter was stripped and hybridized with anti-Ret antibodies (lower panel). (B) Cell lysates from cells treated as in panel (A) were immunoblotted with anti-phosphoERK antibodies (upper panel); the same filter was stripped and hybridized with anti-ERK antibodies (lower panel). (C) EC-Ret^{wt} and EC-Ret$^{1–387}$ were treated for 10 min at 37 °C in RPMI medium (2 μg/ml final concentrations) and then loaded onto a Superdex 200 High Load gel filtration column. The molecular sizes of EC-Ret^{wt} and EC-Ret$^{1–387}$ are indicated.

differentiation. To this aim, we measured the GDNF-dependent neurite outgrowth in PC12-α1/wt cells that stably express both human Ret and GFRα1. As shown in Fig. 5A and B, cells extended long neuritic processes in response to 1-day exposure to GDNF (b) in comparison with the nonstimulated control cells (a). Treatment of the cells with EC-Ret^{wt} (c) but not with EC-Ret$^{1–387}$ truncated protein (d) significantly reduced the number and length of neurites. To further monitor the cell differentiation, we determined the extent of induction of VGF by GDNF stimulation. Consistently with the phenotypical effects reported above,

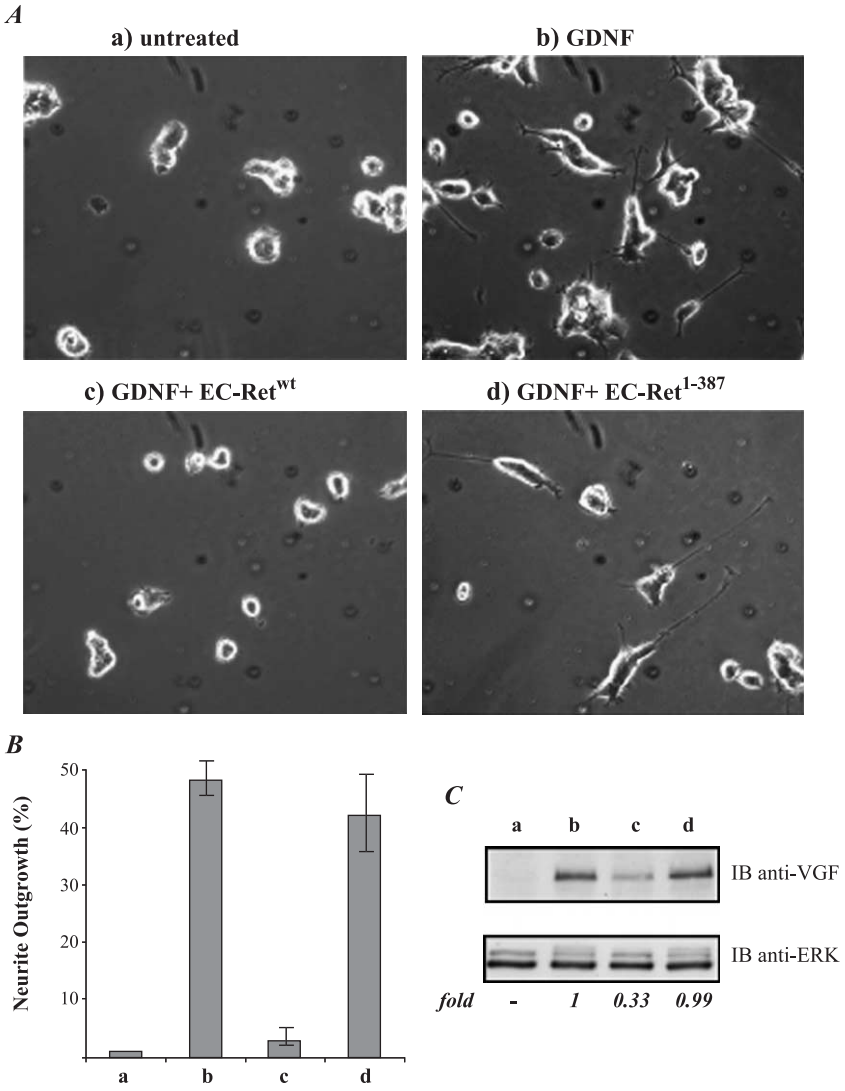

Fig. 5. Effect of EC-Ret proteins on GDNF-induced differentiation of PC12-α1/wt cells. (A) Cells were left nonstimulated (a) or stimulated with GDNF alone (b) or together with EC-Ret^{wt} or EC-Ret$^{1–387}$ proteins (c and d, respectively). Following 24 h of GDNF treatment, the percentage of neurite outgrowth was calculated. (B) The data were expressed as percentage of neurite-bearing cells/total cells counted. Each experiment was repeated a minimum of three times. (C) Cell lysates were immunoblotted with anti-VGF and, to confirm equal loading, with anti-ERK antibodies as indicated. The fold values over control set to 1 (lane b) have been calculated using the NIH Image Program.

treatment with EC-Ret^{wt}, but not with the EC-Ret$^{1–387}$ protein, strongly inhibits the GDNF-dependent stimulation of VGF expression (Fig. 5C).

4. Discussion

Given the obvious difficulty to solve the 3D structure of the Ret/GFRα1/GDNF ternary complex, a need of structural and functional information is still required to identify the regions engaged in the interactions within the complex. The availability of soluble forms of Ret extracellular domains, GDNF and GFRα1 molecules allowed us to directly study the interactions among these components in vitro, using highly homogeneous proteins. We performed cross-linking experiments involving the purified human Ret extracellular portion together with the entire GDNF and GFRα1 molecules followed by MALDI mass spectrometry finger-printing analyses to obtain a direct identification of the chemical linkages joining Ret, the ligand and the coreceptor molecules. In these experiments, we used two recombinant Ret protein forms, the first corresponding to the entire Ret receptor extracellular portion (EC-Ret^{wt}) and the other one to the three first N-terminal domains (CLD1–3) of Ret (EC-Ret$^{1–387}$) lacking the CLD4 and CRD.

The results presented here show that, following cross-linking experiments in the presence of EC-Ret^{wt}, formation of a three-component complex occurred that involved Ret, GDNF and GFRα1 molecules. The pattern of chemical cross-links observed in the analysis of this complex is reported in Fig. 6. On the contrary, formation of the three-component complex could not be detected when the

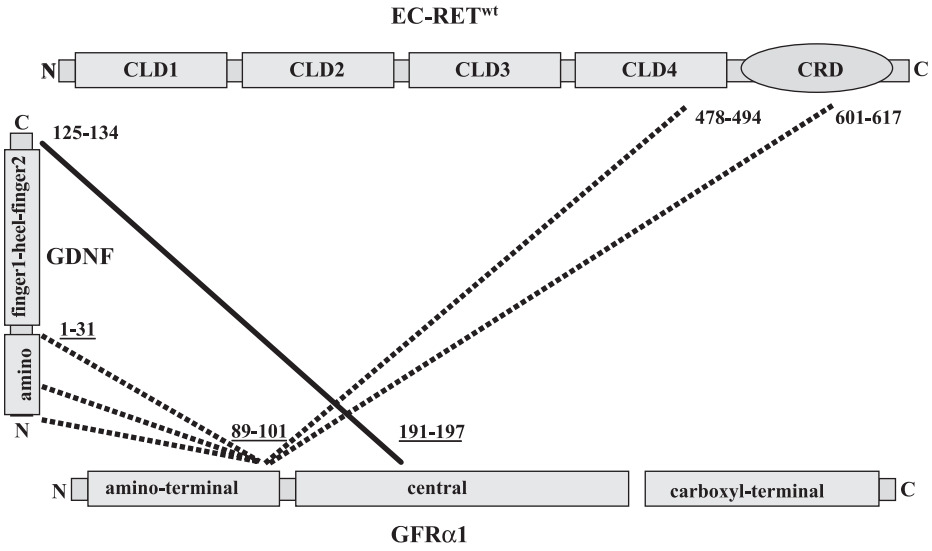

Fig. 6. Schematic representation of covalent cross-links joining GDNF, EC-Ret^{wt} and GFRα1. Lines represent cross-linking bonds; dotted lines indicate that cross-links occur only if EC-Ret^{wt} was present in the complex. Regions concerned by cross-links are indicated by the respective amino acids and are underlined. Domains of GDNF and GFRα1 proteins are labeled accordingly to Refs. [9,10], respectively.

recombinant truncated form of Ret (EC-Ret$^{1–387}$) was used in the experiments. Moreover, formation of two-component complexes involving GDNF and GFRα1 interacting with different stoichiometry was observed in the presence of both native and truncated Ret forms. The panel of covalent linkages found between the ligand and the coreceptor was only slightly different as compared to that observed in the three-component complex. Finally, no complexes between Ret and GDNF or between Ret and GFRα1 could be detected in our analysis.

On the other hand, the current model for GDNF signaling proposes that direct binding of GFRα1 to GDNF is a required event to stimulate Ret and that Ret binding to GFRα1 increases the affinity of GFRα1 for GDNF [8]. Although the absence of complexes between Ret and GDNF could not rule out any substantial physical contacts between these proteins, the data are in good agreement with the need of GFRα1 in the same complex to stimulate Ret activity [8]. Applying a similar logic, it is reasonable to assume either that Ret may bind uniquely to GFRα1 following conformational changes taking place in the coreceptor upon binding GDNF or, alternatively, that a two-component complex of Ret with GFRα1 can take place but at a lower stability with respect to the three-component complex involving Ret, GDNF and GFRα1. In the latter case, it would mean that the affinity of Ret for the preformed GFRα1/GDNF complex is higher than that of Ret for GFRα1 or GDNF kept separately.

As recently reported, the central domain of GFRα1 is necessary to dictate the ligand binding specificity and is critical for GDNF-induced Ret activation [10–12]. In agreement with the reported importance of this central region of GFRα1 for ligand binding, we found that the region 191–197 of GFRα1 is directly joined to GDNF. Interestingly, this GFRα1 region interacts with the 125–134 region in the C-terminal domain of GDNF, which is neighbouring to a surface that includes four negatively charged and four hydrophobic residues. The same region has been reported to form a hot spot for GDNF binding to the GFRα1 coreceptor [8]. Although GFRα1 binds the C-terminal domain of GDNF both in the presence and in the absence of Ret in the complex, an additional contact between the 91–97 region of the GFRα1 molecule and the N-terminal (1–31 residues) of the GDNF molecule occurs exclusively if Ret is present in the same complex (see Fig. 6). This finding suggests that binding of Ret to GFRα1/GDNF complex induces a conformational change in the complex, causing the first residues of the N-terminal region in the GDNF molecule to contact a newly exposed GFRα1 region encompassing residues 91–97. This model is well supported by the assumption that the first 36 residues in the N-terminus of GDNF represent a highly flexible region since they could not be resolved in the crystal structure [26]. This large N-terminal region is well conserved between rat and human, however, despite the high structural homology with neurturin, artemin and persephin, it is unique to GDNF. On the other hand, by transient expression assays, it has been shown to be dispensable for stimulation of Ret downstream signaling [9], thus raising the question of understanding the function for this region in the complex. A plausible interpretation for the results presented suggests that this region could be implicated in the formation of a high-affinity GDNF-binding site on GFRα1 that is observed only in the presence of Ret [7].

The observation that a GFRα1 region (91–97), including residues located in the third predicted α-helix of the N-terminal domain, interacts with GDNF is in apparent discrepancy with the dispensability of this region for GDNF-induced stimulation of Ret. Indeed, variants of the coreceptor molecule truncated of the entire N-terminal domain still retain the ability to bind GDNF although at

low efficiency, thus indicating that this region of GFRα1 is not needed for ligand binding [11]. On the other hand, cross-linking experiments with either of the specific reagent used (BS3 and EDAC) strongly support the relevance of this interaction. However, it should be pointed out that the interaction of the N-terminal portion of GFRα1 with the N-terminal region of GDNF only occurs following formation of the ternary complex in the presence of Ret. On the contrary, the contact between the GFRα1 191–197 region with the GDNF C-terminal portion was observed regardless of the presence of Ret. Therefore, it is reasonable to assume that the N-terminal portion of GFRα1 is required to stabilize the GFRα1/GDNF complex, thus explaining the suboptimal ligand binding efficiency reported for the deletion mutants [11].

When the Ret, GDNF and GFRα1 three-component complex was examined following cross-linking experiments, we found that Ret is covalently linked to GFRα1, but not to GDNF. In particular, the N-terminal portion of GFRα1 joined to the CLD4 and CRD domains, located proximal to the transmembrane domain, was involved in these interactions. On the other hand, no interaction involving the three first CLDs domains of Ret (CLD1–3) was found with neither cross-linking reagents. Consistently, we show that EC-Ret$^{1-387}$ protein, corresponding to the CLD1–3 domains and thus lacking both the CLD4 and CRD, does not bind to GFRα1/GDNF complex since no covalent cross-link involving EC-Ret$^{1-387}$ was found. The structural evidence that CLD4 and CRD are required for binding of Ret to GFRα1/GDNF complex was strongly confirmed by functional data on living cells. Indeed, in PC12-α1/wt cells, the stimulation by GDNF of both Ret intracellular signaling and cell differentiation were strongly inhibited by EC-Ret^{wt}, but not by EC-Ret$^{1-387}$ protein, thus indicating that the truncated EC-Ret$^{1-387}$ that lacks both CLD4 and CRD is not able to act as inhibitory competitor for the membrane-bound Ret receptor.

As a whole, these results demonstrate that CLD4 and CRD are dispensable for the correct folding and maturation of Ret extracellular domain, but they are necessary for both structural and functional binding of Ret to the GFRα1/GDNF complex.

By using the homologue-scanning mutagenesis analysis between the extracellular domains of human and *Xenopus* Ret, it has been reported [13] that the existence of a surface spanning the three N-terminal CLDs (CLD1–3) of human Ret that is required for ligand binding. This finding led to a model that must presuppose a high flexibility of the Ret extracellular portion, allowing Ret to reach the binding sites on GFRα1 anchored to the plasma membrane. A strong implication of our results resides in the possibility to reformulate this interaction model, suggesting that requirement of CLD1–3 elements for binding is rather indirect, while a physical interaction occurs between the C-terminal portion of Ret (CRD and CLD4 domains) and the GFRα1/GDNF complex in close contact with the cell membrane. The first three N-terminal domains of Ret either would contribute to stabilize the complex once formed or might be necessary for Ret to adopt a conformation competent for the assembly of a functional complex.

In conclusion, we show that two small regions of GDNF are engaged in close physical interactions with GFRα1, one of which requires the presence of Ret in the same complex to be positioned close to the GFRα1. Conversely, the complex between Ret and GFRα1/GDNF is stabilized by major contacts involving the N-terminal domain of GFRα1 and the CLD4 and the CRD domains of Ret. These domains are necessary for both structural and functional binding of the receptor to the complex. Results make it possible to suggest a model for the molecular basis of ligand-mediated Ret stimulation in attempts to design minimal agonists of the GDNF molecule to be used both as therapeutics and diagnostic agents for neurodegenerative diseases.

Acknowledgments

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