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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.
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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-

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