The Ret Receptor Protein Tyrosine Kinase Associates with the SH2-containing Adapter Protein Grb10*

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Ret is a receptor protein tyrosine kinase that has been implicated in the development of the enteric nervous, endocrine, and renal systems. Mutations associated with multiple endocrine neoplasia types 2A and 2B (MEN 2A and 2B) have been shown to activate the intrinsic kinase and transforming ability of ret (Santoro, M., Carlomagno, F., Romano, A., Bottaro, D. P., Dathan, N. A., Greico, M., Fusco, A., Vecchio, G., Matoškova, B., Kraus, M. H., and Paolo Di Fiore, P. (1995) Science 267, 381–383). Using the cytoplasmic domain of Ret as bait in a yeast two-hybrid screen of a mouse embryonic library, it was discovered that the src homology 2 (SH2) domain containing protein Grb10 bound Ret. Grb10 belongs to an emerging family of SH2 containing adapter proteins, the prototypical member being Grb7. Using glutathione S-transferase fusion proteins, it was demonstrated that the SH2 domain of Grb10 specifically interacted with Ret. Additionally, when an EGFR RET chimera, it was shown that Grb10 bound Ret in an activation dependent manner in vivo. This is the first description of a receptor protein tyrosine kinase that utilizes Grb10 as a signaling intermediate.

Cells respond to a variety of extracellular stimuli, including nutritional deprivation, osmotic stress, growth factors, and hormones. Many of these responses are mediated by transmembrane receptors and activation of these receptors can trigger cellular proliferation, differentiation, survival, or alterations in metabolism (1, 2). Alteration in protein phosphorylation is the major conduit for the flow of information from a cell’s exterior to the interior. Receptor protein tyrosine kinases (RPTKs) are important mediators of such signals. The ret protooncogene encodes a transmembrane tyrosine kinase receptor with a cadherin and cytokerin-rich extracellular domain and a tyrosine kinase containing intracellular domain (2). Ret was originally identified as a transforming gene detected by transfection of NIH 3T3 cells with DNA from a human T cell lymphoma (3). Subsequently, it was discovered that the activated ret transforming gene was generated during a recombination of its carboxyl-terminal kinase domain with unrelated S’ sequences (4). Ret is found to be Rearranged and constitutively activated in a large proportion of thyroid papillary carcinomas (5–7). Dominant transforming mutations of the ret protooncogene in the germ line have been shown to result in multiple endocrine neoplasia types 2A and 2B (MEN 2A and 2B) (8–11). Studies based on ret expression have suggested that ret may encode the receptor for a factor that is involved in the migration, proliferation, or survival of a variety of neuronal lineages (12). The importance of ret during development is underscored by the finding that mice deficient in the expression of the ret protooncogene display renal agenesis and lack enteric neurons throughout the digestive tract (13).

Since the ligand for Ret has not been identified, studies of the signaling pathways initiated by the ret receptor kinase have been difficult to perform. To overcome this problem, we have used an EGFR/Ret chimera in which the extracellular ligand binding domain of the EGF receptor is fused in frame to the cytoplasmic domain of the Ret RPTK (14). Such a receptor chimera is potently activated by EGF. Additionally, we have employed the yeast two hybrid system to identify downstream signaling molecules engaged by the cytoplasmic domain of Ret. When the Ret cytoplasmic domain was used as bait in the two hybrid system to screen for interacting molecules, a partial cDNA encoding the SH2 domain of Grb10 was isolated that specifically bound Ret. Grb10 is a recently discovered molecule that contains an SH2 domain, a central domain, and a proline-rich region (15). The central domain of Grb10 is similar to the putative Caenorhabditis elegans gene F10E9.6 and contains a pleckstrin homology domain (16, 17). It has been suggested that F10E9.6 represents mig-10, a gene that is crucial for the embryonic migration of a subset of C. elegans neuronal cells (18). The exact function of pleckstrin homology domain is not known, but it has been proposed to be involved in protein-protein or protein-lipid interactions (17, 19, 20). The SH2 domain of Grb10 is 64% identical to the SH2 domain of a previously described gene designated Grb7 (15, 21). SH2 domains have been shown to mediate binding to phosphotyrosines embedded in an appropriate sequence context (22–24). Thus Grb10 and Grb7 define a new family of SH2 domain containing adapter proteins that may serve to link activated receptor tyrosine kinases to downstream signaling molecules.

Grb7 has previously been shown to bind to activated HER2/neu, a receptor with close similarity to the EGF receptor, and was found to be coamplified and overexpressed along with HER2/neu in certain forms of breast cancer (21). Grb10 was cloned by screening a 14q11 expression library with a phosphorylated 32P-labeled carboxyl-terminal segment of the EGF receptor (15). Despite being cloned in this manner, Grb10 has not been shown to bind the EGFR or any other RPTK, in vivo. However, we now show that the SH2 domain of Grb10 specifically associates with the cytoplasmic domain of Ret. Furthermore, using an EGFR/Ret receptor chimera, it was determined...
that Grb10 bound Ret in an activation-dependent fashion in vivo. This is the first identification of a receptor that is engaged by Grb10.

MATERIALS AND METHODS

Yeast Two-hybrid Screen and cDNA Isolation—The cytoplasmic domain (amino acids 660–1115) of the murine Ret RPTK (25) was obtained by polymerase chain reaction using a ret cDNA as template and custom oligonucleotide primers. The sense primer, including a custom SfiI site (underlined) had the sequence: ATACTGGCCATGGAGGCCCACCA-CAAGCATGGGCACAAGCCG and the antisense primer including a custom SalI site had the sequence: GACACTGCGACATGTTAACATATTCAAGGGTGCTACATTTTGC. The amplified fragment was cloned into the yeast bait plasmid pAS1CYH2 and expressed as a hybrid gene consisting of an upstream HA epitope tag and downstream the DNA-binding domain of the modular yeast transcription factor GAL4 (26). This bait plasmid was cotransformed with a day 10.5 mouse embryonic stem cell plasmid consisting of an upstream HA epitope tag and downstream the DNA-binding domain of GAL4 (27). Several of the 10⁶ transformants screened had determined β-galactosidase activity. Library plasmid recovered from the positive clones was used in a cotransformation assay with either the Ret cytoplasmic bait or other control heterologous baits. One cDNA was found to interact specifically with the Ret cytoplasmic domain but not with heterologous baits containing the cytoplasmic domains of the Eck RPTK, Fas, and CD40. The construction of these heterologous baits has been described previously (27, 28).

DNA sequence analysis was carried out on both strands using the Sequenase kit (U. S. Biochemical Corp.) and custom synthetic oligonucleotide primers. Homology searching against GenPept, PIR, and SwissProt data bases was performed using the on-line BLAST network service.

Expression Vectors—Construction of hemagglutinin-tagged Eck cytoplasmic domain (HA-Eck) has been described previously (28). The HA-Ret expression plasmid was constructed in a similar fashion by subcloning a fragment from the yeast bait vector that encoded HA epitope-tagged Ret cytoplasmic domain into the mammalian expression vector pcDNA3. Construction and characterization of the EGFR/Ret construct has been described earlier (14). For these studies, EGFR/Ret was subcloned into the mammalian expression vector p2ev-SV (Invitrogen), where its expression was driven by the SV40 promoter. The Grb10 expression construct (15) was a gift from Ben Margolis (New York University). A20 expression vector was derived by cloning the entire cDNA as template and custom synthetic oligonucleotide primers. The A20 expression construct encoding hemagglutinin-tagged Ret cytoplasmic domain (HA-Eck) has been described previously (28). The HA-Ret expression plasmid was cloned in a similar fashion by subcloning a fragment from the yeast bait vector encoding HA epitope-tagged Ret cytoplasmic domain into the mammalian expression vector pcDNA3.

Production of GST Fusion Proteins—Construction of GST fusions expressing the SH2 domains of Grb2 and PLCγ and p85 has been described earlier (28, 30, 31). The Grb10 SH2 GST fusion protein was made by cloning amino acids 503–622 of Grb10 into the pGST tag vector. GST fusion proteins were prepared using standard procedures and the recombinant proteins immobilized onto glutathione-agarose beads (Sigma).

GST Binding and Coimmunoprecipitation Assays—293T cells were transfected with the HA- or HA-Ret construct, metabolically labeled with 100 μCi/ml of [35S]methionine and cysteine (Tran±S-label, ICN) for 8 h and then lysed in lysis buffer containing 50 m M Tris, pH 7.6, 150 m M NaCl, 1% Nonidet P-40, 6 M urea, 0.1% sodium orthovanadate in the presence of protease inhibitors (5 μg/ml leupeptin, 5 μg/ml aprotinin, 50 μg/ml soybean trypsin inhibitor, and 5 μg/ml pepstatin). Lysates containing an equivalent number of trichloroacetic acid-precipitable counts were incubated with approximately 2 μg of the indicated GST fusion proteins in 1 ml of lysis buffer for 2 h at 4 °C, washed, and bound material eluted by boiling in 1% SDS. The eluates were then diluted 10-fold with lysis buffer and immunoprecipitated with anti-HA monoclonal antibody 12CA5.

293T cells were cotransfected with EGFR/Ret and either Grb10 or A20 expression vectors, starved in 1% BSA for 24 h, and either not treated or treated with 100 ng/ml of EGF for 5 min and lysed in lysis buffer. Cleared cell lysates were incubated with 5 μg of anti-Grb10 (number 369) (15) or anti-A20 (32) for 2 h, followed by incubation with protein A/G, washed, and bound material eluted by boiling in 1% SDS. The eluates were subjected to Western blot analysis as described below.

Western Blot Analysis—Precipitated immune complexes were resolved by SDS-PAGE, transferred to nitrocellulose by electroblotting, blocked with 1% BSA in Tris-buffered saline containing 0.1% Tween (TBS-T) overnight at 4 °C, and then incubated with 4G10 anti-phosphotyrosine monoclonal antibody (Upstate Biotechnology, Inc.), followed by incubation with horseradish peroxidase-conjugated goat anti-mouse or IgG (Bio-Rad). After extensive washing, membranes were developed using a chemiluminescent reaction (ECL, Amersham Corp.) according to the manufacturer’s instructions. Detection of Ret protein was done by resolving whole cell lysates by SDS-PAGE followed by immunoblotting with anti-Ret antibody (14).

RESULTS AND DISCUSSION

The cytoplasmic domain (amino acids 660–1115) of the murine Ret RPTK (25), which when expressed in 293T cells displayed constitutive tyrosine kinase activity (data not shown), was fused in frame to the GAL4 DNA-binding domain in the yeast vector pAS1CYH2. This was used as bait to detect interacting proteins encoded by library cDNAs fused to the VP16 activation domain. The rationale for this strategy was that the truncated cytoplasmic domain of Ret would autophosphorylate itself as a result of constitutive kinase activity and bind downstream signaling molecules in the yeast two-hybrid screen. A total of approximately 10⁶ transformants were screened by expression in a yeast strain harboring lacZ and His3 genes under control of the GAL4 upstream activating sequence. Transformants were plated on histidine-deficient media to select for interacting clones as described previously (28).

One interacting clone that grew on histidine-deficient media and was also strongly positive in the β-galactosidase filter assay was characterized further. As shown in Table I, this clone interacted specifically with the Ret cytoplasmic domain, but not with cytoplasmic domains from the unrelated Eck RPTK, Fas, or CD40. Upon sequencing, this clone was found to encode an SH2 domain (amino acids 466–622) of a recently described protein, Grb10 (15). To obtain independent confirmation of the interaction, cell lysates from cells transfected with an expression construct encoding hemagglutinin-tagged Ret cytoplasmic domain (HA-Ret) were incubated with GST fusions of SH2 domains from Grb10, Grb2, or PLCγ. As shown in Fig. 1, the SH2 domain of Grb10 bound the Ret cytoplasmic domain as did the SH2 domain of Grb2 and the carboxyl-terminal SH2 domain of PLCγ. This binding is presumably mediated by tyrosines that have been autophosphorylated as a result of constitutive kinase activity of the HA-Ret fusion protein. Grb2 and PLCγ have been shown previously to bind to the Ret RPTK in vitro and in vivo, respectively, and therefore served as important positive controls (14, 33). This interaction was specific, since the NH2-terminal SH2 domain of PLCγ or GST by itself did not bind. To determine whether the binding of the Grb10 SH2 domain was specific for Ret, a similar set of experiments was conducted using lysates from cells transfected with the cytoplasmic domain of an unrelated RPTK, Eck. The Eck cyto-
plasmic domain did not bind Grb10, but as reported earlier it did bind the COOH-terminal SH2 domain of the p85 subunit of phosphatidylinositol kinase (28).

Since the ligand for the Ret RPTK has not been identified, the EGFR/Ret chimeric construct was used for in vivo binding studies. In this receptor chimera, the cytoplasmic domain is derived from the Ret RPTK, and its tyrosine kinase activity is increased substantially upon addition of EGFR (14). This increase in catalytic activity of Ret (autoactivation) can be easily assessed by measuring the degree of autophosphorylation. Cells were cotransfected with expression vectors containing EGFR/Ret and Grb10 or an unrelated cytoplasmic protein A20, treated or not treated with EGF, and Grb10 or A20 immunoprecipitates subjected to immunoblotting with an anti-phosphotyrosine antibody (anti-pTyr, top panel). The bottom panel is an immunoblot of respective cell lysates with anti-Ret antibody.

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Fig. 1. SH2 domain of Grb10 specifically binds Ret in vitro. 293T cells were transfected with an expression vector containing either the cytoplasmic domain of Ret that was HA epitope-tagged (HA-Ret) or a similar expression vector containing HA-Eck. Cells were then metabolically labeled, and lysates were incubated with GST fusion proteins as indicated in the figure. Bound material was dissociated by boiling in 1% SDS, diluted, and reimmunoprecipitated with anti-HA antibody.

Fig. 2. Grb10 binds activated Ret RPTK in vivo. 293T cells were cotransfected with expression plasmids containing EGFR/Ret and either Grb10 or A20. After staining in 1% BSA for 24 h, cells were untreated or treated with EGF (100 ng/ml). Anti-Grb10 or anti-A20 immunoprecipitates were run on an SDS-PAGE gel and subjected to immunoblotting with an anti-phosphotyrosine antibody (anti-pTyr, top panel). The bottom panel is an immunoblot of respective cell lysates with anti-Ret antibody.