Direct Association between the Ret Receptor Tyrosine Kinase and the Src Homology 2-containing Adapter Protein Grb7*

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Adapter proteins containing Src homology 2 (SH2) domains link transmembrane receptor protein-tyrosine kinases to downstream signal transducing molecules. A family of SH2 containing adapter proteins including Grb7 and Grb10 has been recently identified. We had previously shown that Grb10 associates with Ret via its SH2 domain in an activation-dependent manner (Pandey, A., Duan, H., Di Fiore, P. P., and Dixit, V. M. (1995) J. Biol. Chem. 270, 21461–21463). We now demonstrate that the related adapter molecule Grb7 also associates with Ret in vitro and in vivo, and that the binding of the SH2 domain of Grb7 to Ret is direct. This binding is dependent upon Ret autophosphorylation since Grb7 is incapable of binding a kinase-defective mutant of Ret. Thus two members of the Grb family, Grb7 and Grb10, likely relay signals emanating from Ret to other, as yet, unidentified targets within the cell.

The ret protooncogene encodes a transmembrane receptor protein-tyrosine kinase (RPTK). The extracellular domain of Ret is unrelated to any other subfamily of RPTKs and contains multiple cadherin-like repeats and a cysteine-rich region (1). Ret plays a critical role in renal development as well as that of endocrine organs derived from the neural crest including the adrenal medulla and the thyroid gland. Mutations in the ret protooncogene have been shown to result in a variety of disorders including Hirschsprung’s disease, multiple endocrine neoplasias 2A and 2B, and familial medullary thyroid carcinoma (2–8). More recently, ret has been shown to be a dominant acting oncogene capable of germline transmission. Knockout studies have demonstrated that mice lacking ret have renal agenesis or severe dysgenesis and lack enteric neurons (9). This observation is crucial for the migration of a subset of neuronal cells and contains a pleckstrin homology domain. The central domains of Grb7 and Grb10 are 54% identical to each other (16). Since Grb10 interacted with the Ret receptor protein-tyrosine kinase in an activation dependent manner, we asked if Grb7 might also associate with Ret in a similar fashion. This reasoning was based on the fact that since the SH2 domains of Grb7 and Grb10 are 64% identical to each other, it was possible that they engaged similar receptor protein-tyrosine kinases. Grb7 has previously been shown to associate with HER2/neu, a receptor closely related to the epidermal growth factor receptor (EGFR) (18). Activation of the HER2 receptor led to association and tyrosine phosphorylation of Grb7 in cells overexpressing the chimeric EGFR/HER2 receptor (18). In this study we demonstrate that Grb7 directly associates with Ret via its SH2 domain, and unlike Grb10, it undergoes tyrosine phosphorylation in response to Ret activation.

MATERIALS AND METHODS

Expression Vectors—Construction of hemaggulinin tagged Ret cytoplasmic domain (HA-Ret) plasmid has been described previously (14). Construction and characterization of the EGFR/Ret construct has also been described earlier (13). For these studies, EGFR/Ret was subcloned into the mammalian expression vector, pZEd5V (Invitrogen) where its expression was driven by the SV40 promoter. The Grb7 expression construct was a gift from Dr. Ben Margolis (University of Michigan). The expression vector for Flag-epitope tagged CD40 binding protein (CD40bp-Flag) has been described earlier (19). Construction of hemaggulitin (HA)-tagged Ret cDNA and the kinase defective mutant encoding methionine instead of lysine at position 758 (K758M) has been described elsewhere (20).

Production of Fusion Proteins—The SH2 domain of Grb7 expressed as a GST fusion was a gift from Dr. Ben Margolis. Construction of GST fusions expressing the NH2-terminal and COOH-terminal domains of PLCγ have been described earlier (21). GST fusion proteins were prepared using standard procedures and the recombinant proteins immobilized onto glutathione-Agarose beads (Sigma). Soluble GST fusions of SH2 Grb7 and Grb10 were generated by eluting the beads with 10 mM reduced glutathione followed by dialyzing the eluate against phosphate-buffered saline. His-tagged constructs were generated by cloning the SH2 domains of Grb7 (amin acids 434–535 of mouse Grb7) and Grb10 (amin acids 503–622 of mouse Grb10) into the pET15b vector, and the COOH-terminal SH2 domain of PLCγ (amin acids 668–756 of bovine PLCγ) into the pQE-30 vector. The His-tagged fusion proteins were purified using Ni2+-NTA agarose beads (Qiagen) and eluted with imidazole according to manufacturer’s instructions.

The abbreviations used are: RPTK, receptor protein tyrosine kinase; GST, glutathione S-transferase; SH, Src homology; EGFR, epidermal growth factor; ERGFR, epidermal growth factor receptor; HA, hemaggulitin; PLCγ, phospholipase Cγ; PAGE, polyacrylamide gel electrophoresis; BSA, bovine serum albumin.

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Grb7 Associates with the Ret Receptor Tyrosine Kinase

GST Binding and Competition Assays—Direct binding of the SH2 domain of Grb7 to activated EGFR/Ret was evaluated as follows. First, 293 cells were transfected with EGFR/Ret, and after 48 h, starved overnight in 1% BSA. Anti-EGFR immunoprecipitates from EGFr-treated or untreated lysates were resolved by SDS-PAGE and transferred onto nitrocellulose. The nitrocellulose membrane was then incubated with 5 μg of soluble GST fusion of SH2 domains for 1 h at room temperature. The samples were then incubated with 5 μg of soluble GST fusion of SH2 domains for 30 min at room temperature. The samples were then incubated with 5 μg of soluble GST fusion of SH2 domains for 1 h at room temperature followed by washing and SDS-PAGE. The gels were transferred onto nitrocellulose, and immunoblotted with an anti-GST monoclonal antibody (Santa Cruz Biotechnology, Inc., CA) to detect the bound SH2 domain GST fusion protein.

Communoprecipitation Assays—In vivo binding studies, 293 cells were cotransfected with EGFR/Ret and either Grb7 or CD40bp expression constructs, or Grb7 alone, starved in 1% BSA overnight, and either not treated or treated with 100 ng/ml EGF for 5 min and lysed in lysis buffer. Cleared cell lysates were incubated with 5 μg of anti-Grb7 (N-20) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) or anti-Flag monoclonal antibody for 2 h at 4°C, followed by incubation with protein A/G, and washed and bound material was eluted by boiling in sample buffer. The eluates were subjected to Western blot analysis as described below.

For in vivo binding studies using SKBR-3 cells, these cells were transiently transfected with EGFR/Ret using the calcium phosphate method. 48 h after transfection, cells were starved in 1% BSA overnight and left untreated or treated with EGF followed by lysis as described above. The lysates were immunoprecipitated with anti-Grb7 (N-20) (Santa Cruz Biotechnology, Inc.) followed by Western blot analysis with anti-phosphotyrosine 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 40 μg anti-phosphotyrosine monoclonal antibody (Upstate Biotechnology, Inc.), followed by incubation with horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit IgG (Bio-Rad). After extensive washing, membranes were developed using a chemiluminescent reaction (Boehringer Mannheim) according to the manufacturer’s instructions. Detection of EGFR/Ret protein was done by immunoprecipitating parallel samples with anti-human EGFR monoclonal antibody (Upstate Biotechnology, Inc.) followed by immunoblotting with the same antibody.

RESULTS AND DISCUSSION

Given the importance of Ret in mammalian development and oncogenesis, characterization of molecules that participate in its signaling is of paramount importance. Since the ligand for Ret is unidentified, a strategy was utilized to exploit the finding that the cytoplasmic domain of Ret mimics the activated form of the receptor as it possesses constitutive tyrosine kinase activity that results in autophosphorylation. The phosphorylated tyrosines in turn, should be capable of engaging downstream signaling molecules. Using the Ret cytoplasmic domain fused to the GAL4 DNA binding domain as bait in the yeast two-hybrid screen, we had previously identified Grb7, an SH2-containing adapter protein, as a downstream target of Ret (14). Grb10 belongs to an emerging family of SH2 containing adapter proteins with Grb7 as the prototypical member (15–17). Both of these proteins contain a single SH2 domain at the COOH terminus, a central region containing a pleckstrin homology domain, and a proline-rich region at the NH2 terminus.

Grb7 was initially identified by screening a 3'gt11 expression library with a phosphorylated 32P-labeled carboxyl-terminal segment of EGFR (17). Despite this, it has not been shown to bind to EGFR in vivo. It does, however, associate with HER2/
which expresses endogenous Grb7 was used for analysis. This cell line was transiently transfected with the EGFR/Ret plasmid, and left untreated or treated with EGF. Anti-Grb7 immunoprecipitates were then immunoblotted with an anti-phosphotyrosine antibody to detect co-precipitated EGFR/Ret. As shown in Fig. 2C, the endogenous Grb7 again associated with the activated chimeric Ret receptor but not with the unactivated receptor. There was equal expression of EGFR/Ret in the unactivated and activated samples (data not shown).

The SH2 domain of Grb7 could either bind the autophosphorylated Ret receptor directly, or this binding could be mediated by adapter proteins such as Shc which are phosphorylated by Ret. In order to distinguish between these two possibilities, a direct binding assay using a soluble GST fusion of the SH2 domain of Grb7 was carried out. EGFR/Ret was immunoprecipitated from transfected cells that were either untreated or treated with EGF and the immunoprecipitates were resolved by SDS-PAGE followed by transfer onto a nitrocellulose membrane. This membrane was then incubated with soluble SH2 GST Grb7 followed by conventional immunoblotting with an anti-GST monoclonal antibody to detect the GST fusion bound to immunoprecipitated EGFR/Ret receptor. As shown in Fig. 3A, SH2 GST Grb7 directly bound the activated chimeric receptor on the membrane. This confirms that Grb7 does not need an intermediate molecule to bind to the activated Ret receptor.

In order to compare the binding abilities of Grb7 and Grb10, in vitro experiments utilizing soluble forms of His-tagged and GST-tagged SH2 Grb7 and Grb10 were carried out. EGFR/Ret receptors were immunoprecipitated and phosphorylated in vitro, and then incubated with soluble GST fusions of the SH2 domains. In parallel competition experiments, the samples were preincubated with increasing concentrations of His-
Grb7 Associates with the Ret Receptor Tyrosine Kinase

FIG. 3. Grb7 interacts directly with Ret, and Grb10 competes with Grb7 for binding to Ret. A, immunoprecipitated EGF/Ret from 293 cells treated as indicated was resolved by SDS-PAGE and transferred onto nitrocellulose. The membrane was incubated with 5 μg/ml soluble SH2 Grb7 GST followed by immunoblotting with anti-GST antibody. The arrow indicates the position of EGF/Ret where bound SH2 Grb7 can be detected. B, in vitro competition assay. In vitro phosphorylated EGF/Ret receptor was incubated with soluble SH2 Grb7 GST with or without a preincubation with the indicated excess of soluble His-tagged Grb10 or COOH-terminal SH2 PLCγ. The blot was immunoblotted with an anti-GST antibody to detect bound Grb7. C, same as panel B except that soluble SH2 Grb10 GST was used instead of SH2 Grb7 GST, and His-tagged SH2 Grb7 was used for competition.

In summary, we have shown that Grb7 associates with the Ret receptor tyrosine kinase through its SH2 domain and that activation of Ret results in tyrosine phosphorylation of Grb7. Given the importance of Ret during development and in carcinogenesis, signaling molecules downstream of Ret are likely to be important as they may mediate some of these actions of Ret. Clearly, determining the binding sites of Grb7 and Grb10 will be important to further dissection of the signaling pathways initiated by Ret. However, it should be noted that it has been exceedingly difficult to map the Grb7 binding sites on the HER2/neu receptor (18). Nevertheless, studies are ongoing in our laboratories to delineate the site(s) on the Ret receptor protein-tyrosine kinase responsible for binding to these adapter molecules. Further, it should be noted that though Grb7 is tyrosine phosphorylated in response to Ret activation, we have failed to see a similar phosphorylation in the case of Grb10. However, the significance of this phosphorylation is not clear at the present time. One explanation may be that this difference in tyrosine phosphorylation may serve to recruit distinct downstream effectors. Identification of these downstream effectors will be important in dissecting Ret signaling pathways.

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