Mitogenic Signaling by Ret/ptc2 Requires Association with Enigma via a LIM Domain*

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The ret/ptc2 papillary thyroid cancer oncogene, an oncogenic form of the c-ret receptor tyrosine kinase, is the product of a somatic crossover event fusing the dimerization domain of the type Iα regulatory subunit of cyclic AMP-dependent protein kinase (R1) with the tyrosine kinase domain of c-ret. Mitogenic activity of Ret/ptc2 required dimerization via the N terminus of R1 and a tyrosine residue located C-terminal to the kinase core of Ret, Tyr-586 (Durick, K., Yao, V. J., Borrello, M. G., Bongarzone, I., Pierotti, M. A. and Taylor, S. S. (1995) J. Biol. Chem. 270, 24642-24645). Using the yeast two-hybrid system, Ret/ptc2 binding proteins were identified, and the sites of interaction with Ret/ptc2 were mapped. The SH2 domains of phospholipase Cγ and Grb10 were both identified, and binding depended on phosphorylation of Tyr-539 and Tyr-429, respectively. These interactions, however, were not required for mitogenic signaling. The second of the three LIM domains in Enigma (Wu, R. Y., and Gill, G. N. (1994) J. Biol. Chem. 269, 25085-25090) was also identified as a Ret/ptc2 binding domain. Enigma, a 455-residue protein, was discovered based on its interaction with the insulin receptor through the C-terminal LIM domain. Although the association with Enigma required Tyr-586 of Ret/ptc2, the interaction was phosphorylation-independent. In contrast to the SH2 interactions, disruption of the interaction with Enigma abolished Ret/ptc2 mitogenic signaling, suggesting that LIM domain recognition of an unphosphorylated tyrosine-based motif is required for Ret signal transduction.

The c-ret proto-oncogene encodes a receptor tyrosine kinase with a cadherin-like extracellular domain (1). Mutations of c-ret are responsible for two distinct classes of genetic disease. Germline loss of function mutations in c-ret result in the developmental disorder Hirschsprung's disease (2, 3), while activating mutations result in the multiple endocrine neoplasia family of inherited cancers (4–7). In addition to the germline alterations, somatic mutational events lead to constitutively active forms of c-ret, and these are found in nearly half of all papillary type thyroid carcinomas (8).

Chromosomal translocations or inversions in papillary thyroid carcinoma (ptc) give rise to various fusion proteins where the C-terminal tyrosine kinase domain of c-ret is fused to an N-terminal portion of another gene product. One of the resultant transforming proteins, observed in multiple independent cases of ptc, was the product of a crossover between the type Iα regulatory subunit of cyclic AMP-dependent protein kinase (R1) gene with c-ret (9). This protein, Ret/ptc2, is 596 residues in length and contains the N-terminal two-thirds of R1 followed by the entire tyrosine kinase domain of c-ret (10). Using a microinjection-based assay for mitogenic activity, we previously showed that the N-terminal dimerization domain of Ret was essential for constitutive activation of Ret/ptc2 (11).

In the absence of a known ligand, studies of Ret signaling have been done using activated forms of the Ret tyrosine kinase, like Ret/ptc2, or a chimeric epidermal growth factor/Ret kinase receptor. It was suggested from work with the EGF/Ret chimera that Ret couples to a novel mitogenic signaling pathway because, while growth stimulatory effects were as strong as those of the EGF or platelet-derived growth factor receptors, stimulation of mitogen-activated protein kinases and PLCγ by Ret was, in comparison, very weak (12). Work with Ret/ptc2 indicated that both intrinsic protein tyrosine kinase activity and a tyrosine residue (Tyr-586) located outside the kinase core were absolutely required for Ret/ptc2-induced mitogenesis (11).

A yeast two-hybrid screen was used to identify the presumed Src homology 2 (SH2) or phosphotyrosine binding domain/Ret kinase receptor. It was suggested from work with the EGF/Ret chimera that Ret couples to a novel mitogenic signaling pathway because, while growth stimulatory effects were as strong as those of the EGF or platelet-derived growth factor receptors, stimulation of mitogen-activated protein kinases and PLCγ by Ret was, in comparison, very weak (12). Work with Ret/ptc2 indicated that both intrinsic protein tyrosine kinase activity and a tyrosine residue (Tyr-586) located outside the kinase core were absolutely required for Ret/ptc2-induced mitogenesis (11).

EXPERIMENTAL PROCEDURES

Two-hybrid Screen—A yeast two-hybrid screen was performed by the methods of Vogt et al. (14), with reagents from Stan Hollenberg. Ret/ptc2 cDNA was subcloned into the LexA-fusion vector pBTM116 and coexpressed in the L40 strain of Saccharomyces cerevisiae with an embryonic mouse random primed cDNA library. From approximately two million co-transformants, seven interacted specifically with the Ret portion of Ret/ptc2. The cDNA inserts of these were sequenced by the dideoxy method (15), and sequences obtained were compared with the contents of GenBank using the BLAST program through the NIH/NCBI.
server on the World Wide Web. Three library vectors encoded the following mouse sequences: the C-terminal 155 residues of Grb10 (16); 156 residues that share 97% identity with residues 537–693 of rat PLC\(\gamma\) 2 (17); and 131 residues with 95% identity to the C-terminal 131 residues of human Enigma containing all of LIM2 and LIM3 (13).

\textit{\`{e}Galactosidase Assay—}Two hybrid transformants were assayed for \(\beta\)-galactosidase activity by solution assay (18). Units of activity were calculated as: 

\[
\text{activity} = \frac{1750(A_{600})}{(\text{time in min})(\text{volume of culture in assay})(A_{600} \text{ of culture})}
\]

\text{GST-Fusion Affinity Precipitation—}Two-hybrid results were verified using a stably transfected NIH3T3 cell line expressing an EGFR/Ret chimeric protein (12). These cells were treated with 100 nM EGF for 10 min before resuspension in lysis buffer (50 mM HEPES, pH 7.4, 150 mM NaCl, 5 mM KCl, 1 mM CaCl\(_2\), 1 mM MgSO\(_4\), 10% glycerol, 1% Triton, 1 mM benzamidine, 1 mM tosylphenylalanyl chloromethyl ketone, 1 mM N\textsuperscript{\alpha}-p-tosyl-L-lysine chloromethyl ketone, 1 mM phenylmethyalsulfonyl fluoride, 1 mM NaVO\(_4\)). Cleared lysates were incubated for 2 h with 2 \(\mu\)g of GST-fusion protein bound to glutathione-agarose beads in a total volume of 300 \(\mu\)l. The beads were washed four times with lysis buffer, resuspended in SDS-polyacrylamide gel electrophoresis sample buffer, boiled, and run on 7% gels. Proteins were transferred to polyvinylidene difluoride membranes and probed with either rabbit anti-Ret (11) (1:100,000) or anti-phosphotyrosine (1:2500, Transduction Laboratories) antibodies. The GST-fusion proteins used were bacterially expressed from pGEX vectors coding for the following: GST, empty vector; GST-Grb2, murine Grb2 SH2 domain; GST-Enigma, human Enigma LIM2 domain (residues 334–394); GST-Src, murine v-Src SH2 domain; GST-PLC\(\gamma\), murine PLC\(\gamma\) SH2 domain obtained from the two-hybrid screen; GST-Grb10, murine Grb10 SH2 domain obtained from the two-hybrid screen.

\text{Microinjection Mitogenic Activity Assay—}The development of this assay is described in detail elsewhere (11). Briefly, mouse 10T\(\frac{1}{2}\) fibroblasts were plated on glass coverslips and grown to 70% confluence in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. The coverslips were then transferred to Dulbecco's modified Eagle's medium containing 0.05% calf serum. After 24 h of serum starvation, the cells were injected into their nuclei with solutions of injection buffer (20 mM Tris, pH 7.2, 2 mM MgCl\(_2\), 0.1 mM EDTA, 20 mM NaCl) containing 100 \(\mu\)g/ml Ret/ptc2 expression plasmid DNA and 8 mg/ml rabbit IgG (Sigma). For co-injection experiments, 200 \(\mu\)g/ml of a second expression plasmid was also present. All microinjection experiments were performed using an automatic micromanipulator (Eppendorf, Fremont, CA), with glass needles pulled on a vertical pipette puller (Kopf, Tusujng, CA). Entry into S-phase was assessed through incorporation of the thymidine analog 5-bromodeoxyuridine and its subsequent detection by immunostaining. Injected cells were identified by immunostaining of the rabbit IgG injection marker.

\section*{RESULTS}

\text{Yeast Two-hybrid Interactions—}To search for proteins that interact with the Ret/ptc2 oncogenic protein, a mouse random-primed cDNA library was screened using a yeast two-hybrid system (14). Three sequences isolated from the library by interaction with Ret/ptc2 matched the SH2 domain of Grb10, the first SH2 domain of PLC\(\gamma\), and a C-terminal fragment of Enigma that contained LIM domains 2 and 3 (LIM2/3). The interaction of Ret with PLC\(\gamma\) and Grb10 has been observed previously (12, 19). Using the two-hybrid system, the interactions of these proteins with Ret were characterized.

Mutants of Ret/ptc2 were prepared and interactions were quantitated by \(\beta\)-galactosidase activity (Fig. 1b). The SH2 domains failed to interact with a kinase-inactive mutant of Ret/ptc2 (K282R), indicating that these interactions depended on autophosphorylated tyrosine residues. In contrast to the SH2 domains, binding of the LIM2/3 was not diminished in the kinase-inactive mutant. By testing for interaction between the SH2 domains and various Tyr to Phe mutants of Ret/ptc2,
residues required for Grb10 and PLCγ binding were identified as Tyr-429 and Tyr-539, respectively. Both the Grb10 and PLCγ SH2 domains bound to the Y586F mutant, but binding to the Enigma LIM domains was eliminated by this mutation and by a mutation in which the C-terminal 23 residues of Ret were deleted (C574).

**In Vitro Binding to Ret**—To verify the two-hybrid results, affinity precipitation experiments were performed. The binding domains from PLCγ, Grb10, and Enigma were expressed in Escherichia coli as GST-fusion proteins. The three GST-fusion proteins were incubated with lysates of NIH 3T3 cells expressing the EGFR/Ret chimeric receptor (Fig. 1a), where EGFR-dependent activation of EGFR/Ret has been characterized (12). In each case binding to EGFR/Ret from lysates of EGFR-treated cells was observed (Fig. 1c). Results shown were using a GST-fusion protein of only LIM2 from Enigma, because Enigma binding to Ret was determined to be mediated by LIM2.

**Fig. 2. Requirements for the association between Ret/ptc2 and Enigma.** a, mapping of binding determinants in Ret/ptc2. Various fragments of Ret/ptc2, shown schematically, were used in the yeast two-hybrid system to measure interaction with the product of a plasmid expressing full-length Enigma. β-Galactosidase activity of transformants was measured by solution assay, and values shown are averages of duplicate assays. The same pattern was observed in results from three separate experiments with full-length Enigma, the C-terminal half containing the LIM domains, or LIM2 alone. b, tyrosine autophosphorylation of Ret/ptc2 mutants. Lysates of yeast transformed with plasmids expressing fragments shown in panel a were run on 10% SDS-polyacrylamide gel electrophoresis, blotted to nitrocellulose, and probed as in Fig. 1c. Anti-PY, anti-phosphotyrosine. c, specificity of the LIM domains of Enigma for Ret/ptc2. GST-G4–5 plasmids coding for full-length CRP, the three LIM domains of Zyxin (residues 339–542), and the three LIM domains of Enigma (residues 275–455) were co-transformed into yeast with either Ret/ptc2, the pEG202 plasmid expressing the Ret kinase with an intact C terminus, or EGFRtk, a pEG202 plasmid containing as its unique restriction sites the three LIM domains of Zyxin, residues 339–452. In each case binding to EGFR/Ret from lysates of EGFR-treated cells was observed (Fig. 1c). Results shown were using a GST-fusion protein of only LIM2 from Enigma, because Enigma binding to Ret was determined to be mediated by LIM2.2 GST-fusion proteins with the SH2 domains of Grb2 and v-Src were also expressed and tested for in vitro binding. Neither GST alone nor GST-Grb2 bound to the EGFR/Ret chimera. The SH2 domain of v-Src, however, did interact with EGFR/Ret, and interaction with all three GST-SH2 domains required EGFR-stimulated receptor autophosphorylation. In contrast, interaction with GST-LIM2 of Enigma did not require receptor autophosphorylation. Grb2 served as a negative control because Ret has two splice isoforms (10). The long form binds to Grb2 (20) while the short form, used in all of the constructs described here, does not contain the Grb2 consensus site. Both isoforms of Ret are mitogenic (12).

Characterization of the Ret–Enigma Interaction—Because the LIM2 domain of Enigma bound at a site crucial for the mitogenic activity of Ret, this interaction was investigated further. Using an inducible two-hybrid system (21, 22), where higher expression levels were achieved, it was possible to observe the phosphorylation state of Ret using anti-phosphotyrosine.
Zn2⁺, and are found in a variety of homeodomain proteins (24), cytoskeleton-associated proteins (23, 25), protein kinases, and proteins of unknown function (26). Enigma was originally discovered as a protein that binds to exon 16 of the insulin receptor (13) at a tyrosine-based sequence important for receptor internalization, and that interaction is through the

C-terminal LIM domain of Enigma, LIM3. In the present study, Enigma bound to Ret via the LIM2 domain to a sequence required for mitogenic signaling. LIM3 of Enigma is highly specific for the Tyr-based motif in the insulin receptor whereas LIM2 is highly specific for the Tyr-S86-based motif in Ret. The N-terminal portion of Enigma is required for mitogenic signaling because co-injection of only the LIM domains with Ret/ptc2 ablated the mitogenic signal, while co-injection with full-length Enigma did not.

As shown here, Enigma is required for Ret/ptc2 mitogenic signaling while previous results established that Ret tyrosine kinase activity was also required (11). Kinase activity, however, is not required for recruitment of Enigma to Ret/ptc2. Enigma binds to a tyrosine-containing sequence in an activation-independent manner, and this is clearly different from SH2 or phosphotyrosine binding domain interactions. Enigma might either become phosphorylated upon Ret activation or serve to localize Ret to a subcellular position required for kinase-mediated signaling. In either case, these results define a novel mechanism for mitogenic signaling.

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