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13. ABSTRACT *(Maximum 200)*

Ret/ptc2 is a soluble, constitutively active oncogene isolated from human papillary thyroid carcinomas. Sequence analysis of *ret/ptc2* indicated that the gene resulted from a reciprocal rearrangement event between the cAMP-dependent protein kinase regulatory subunit Iα (RIα) and the entire tyrosine kinase domain of the Ret receptor. Using the crystal structure of the insulin receptor tyrosine kinase, we have obtained a working model of the Ret/ptc2 kinase domain. This model was used in conjunction with a microinjection assay and a yeast 2-hybrid screen to: 1) determine that the RIα dimerization domain is critical for eliciting the Ret/ptc2 mitogenic response, and 2) to identify a number of tyrosine residues of Ret/ptc2 which are both autophosphorylated and interact with downstream signaling proteins containing src-homology domains such as Grb10, PLCγ, and a LIM domain containing protein, Enigma. Peptide substrates containing these putative phosphorylated tyrosine residues were synthesized and tested in an *in vitro* kinase assay using Ret/ptc2 expressed in human kidney 293 cells. We have expressed *ret/ptc2* in a bacterial expression system to study and characterize the physical properties of the purified protein. In addition we have made chimeras of the epidermal growth factor receptor and insulin receptor which mimic the *ret/ptc2* gene to test whether the dimerization domain of RIα can activate other receptor tyrosine kinases.

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INTRODUCTION

Protein phosphorylation is probably the most important mechanism for regulation in eukaryotic cells. The tightly regulated enzymes that catalyze the phosphorylation of proteins, the protein kinases, are important components of signaling pathways that regulate normal cellular functions such as the cell cycle, metabolism, differentiation, memory and response to hormones, to name only a few. Over 400 are now known (1), and mutations that generate unregulated or constitutively activated protein kinases are typically oncogenic.

One of the simplest members of the protein kinase family is cAMP-dependent protein kinase, cAPK (2). Being one of the best understood members of the protein kinase family, cAPK also serves as a template for the others since all of these enzymes have evolved from a common ancestor and contain a conserved catalytic core. cAPK, in the absence of cAMP, contains two types of subunits, a regulatory (R) subunit and a catalytic subunit (C). The R_2C_2 holoenzyme is catalytically inactive. In the presence of cAMP the complex dissociates into an R_2-$(cAMP)_4$ dimer and 2 free and active C-subunits. The crystal structure of the C-subunit, solved in our laboratory, serves as a structural template for the entire family of protein kinases (3). It defines the folding of the polypeptide chain as well as the positions of the invariant residues that mostly cluster around the active site (4).

The objective of this grant is to characterize a novel oncogenic tyrosine kinase, Ret/ptc2, found in human papillary thyroid carcinomas. Specifically, we want to understand the molecular basis for its constitutive activation and the basis for its oncogenic properties. Ret/ptc2 is a rearranged gene product composed of the cAMP-dependent protein kinase (cAPK) regulatory subunit Iα (RIα) at its N-terminus fused to the tyrosine kinase core of the Ret proto-oncogene.

Ret Proto-Oncogene

The *ret* proto-oncogene (*proto-ret*) was cloned from a THP-1 human monocytic leukemia cDNA library, and is expressed in a number of human neuroblastoma and leukemia cell lines as 140-190 kDa glycoproteins (5). Sequence analysis identified it to be a member of the receptor tyrosine kinases, and *in situ* hybridization and knockout experiments indicated that *proto-ret* expression is important for neurogenesis, and kidney organogenesis in the mouse (6-8). The ligand for Proto-ret was recently reported as the glial-derived neurotrophic factor (9, 10), and knockout experiments of the gene encoding the ligand yield similar results. Chromosomal rearrangements of human *proto-ret* with other genes and point mutations of *proto-ret* have been linked to a number of human cancers.

The Ret Oncogene Family

The family of *ret* oncogenes can be divided into three separate classes. The first class of *ret* oncogenes was produced *in vitro* by transfecting NIH3T3 cells with high molecular weight DNA from human cell lymphomas (11), human colon carcinoma (12), and human stomach cancer tissue (13). The high propensity of *proto-ret* to rearrange with other genes is reflected in its name for *re*arranged upon *t*ransfection (11).

The second class consists of missense mutations and truncated forms of *proto-ret* that are proposed to result in either hyper- or hypoactivity. These are associated with three dominantly inherited human cancer syndromes: MEN 2A (14), MEN 2B, familial medullary thyroid carcinoma (FMTC) (15, 16), and Hirschsprung's disease (17, 18).

The third class of *ret* oncogenes, isolated from human papillary thyroid carcinomas (19-22), consists of 3 types: *ret/ptc1*, *ret/ptc2* and *ret/ptc3*. The 5' end of the each oncogene is a portion of an unrelated gene fused in frame to the identical splice site of the *proto-ret* gene resulting in an intact functional Ret kinase.

The *ret/ptc1* and *ret/ptc2* oncogenes each produce 2 isoforms as a result of alternative splicing, and unlike proto-Ret are completely cytosolic, phosphorylated on tyrosine residues, and constitutively active (21). The 5' end of *ret/ptc1* is a fragment of a new gene designated, H4(DS10S170) (20, 23), and the 5' end of *ret/ptc3* encodes a gene designated *ele1* or ret fused gene, whose gene product does not show sequence identity to known proteins (22, 24, 25). Unlike *ret/ptc1* and *ret/ptc3*, the N-terminal sequence of *ret/ptc2* gene (21) encodes approximately 60% of a biochemically well characterized protein, the RIα subunit of cAPK. Comparison of Proto-ret, Ret/ptc2 , and the cAPK RIα-subunit is illustrated in Figure 1.

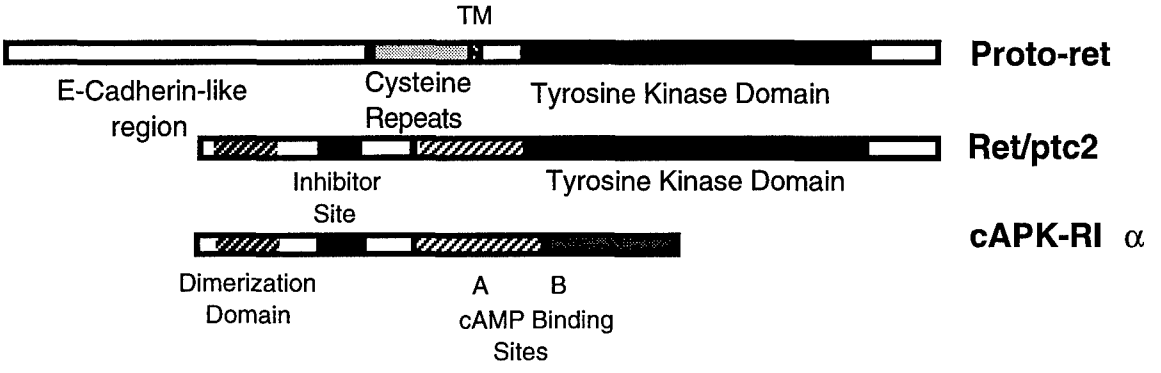

Figure 1. Comparison of Proto-ret, Ret/ptc2, and the cAPK RIα subunit

Regulatory Subunit of cAPK

The regulatory subunit of cAPK maintains the C-subunit in an inactive state by forming a stable R_2C_2 tetramer. A pseudo-substrate inhibition sequence in the R-subunit mimics peptide substrates and fills the peptide binding site of the C-subunit. The binding of cAMP to R causes the complex to dissociate and to release two active C-subunits.

Although there are several unique gene products in the R-subunit family, all share a well-defined domain structure. The RIα subunit begins with a dimerization domain close to its amino terminus followed by a pseudo-substrate inhibitory region and ends with two tandem cAMP binding domains. The RIα dimerization domain is stabilized by two antiparallel interchain disulfide bonds (26). Circular

dichroism studies of the proteolytically isolated RIα dimerization domain indicate it is predominantly α-helical, and extremely stable to thermal denaturation (27). The crystal structure of a deletion mutant of the bovine RIα was recently solved in our laboratory (28). The splice site of the RIα fragment in Ret/ptc2 is at the beginning of the αC-A helix, thus deleting the last 21% of the A site cAMP-binding domain and the entire B-site cAMP binding domain.

SPECIFIC AIMS

Our overall long-term goals are to understand the molecular basis for the constitutive activation of Ret/ptc2 and to characterize the physiological functioning of both *ret/ptc2* and *proto-ret*. Our specific aims are the following:

1. To model the kinase core of Ret/ptc2 based on the crystal structure of the C-subunit of cAPK and on the kinase domain of the insulin receptor.

2. To understand the structural features of Ret/ptc2 that are required for its oncogenic properties. To achieve this we have developed an *in vivo* assay to measure a mitogenic response of *ret/ptc2* by microinjection into nuclei of mouse fibroblast 10T1/2 cells.

3. To identify Ret/ptc2 binding proteins using a yeast two-hybrid system.

4. To characterize the biochemical properties of Ret/ptc2 by overexpressing the protein in *E. coli* and human kidney 293 cells. Phosphorylation sites will be mapped and kinetic properties characterized.

5. To construct homologs of Ret/ptc2 using the tyrosine kinase domains of the EGF receptor and the insulin receptor.

We have made excellent progress in each of these areas, as indicated below, during the past two and a half years. Some of our work has now been published, however since a great deal is still unpublished, we shall describe our ongoing work in some detail. This is an entirely new project for our laboratory but it has become a major focus.

MATERIALS AND METHODS

Model of the Ret/ptc2 Tyrosine Kinase

The computer model of the Ret/ptc2 tyrosine kinase domain was generated using the Insight II software package on a Silicon Graphics Workstation. The model is based on the crystal structure of the insulin receptor tyrosine kinase domain (29).

Construction of Mammalian Expression Plasmids

The cDNA coding for wild-type Ret/ptc2 was excised from a pMAM-neo expression vector previously described (21) using the restriction enzyme *Xba*I. This fragment was subcloned into the *Xba*I restriction site of the pRc/CMV mammalian expression vector (Invitrogen). Restriction digests were performed to screen for orientation, and then the entire cDNA sequence was verified using dideoxy sequencing (30) with Sequenase v. 2.0 (U.S. Biochemicals Corp.). Site-directed mutagenesis was performed by the Kunkle method (31) using the Mutagene kit (Bio-Rad). Constructs expressing deletion mutants of Ret/ptc2 were made by introducing *Nhe*I restriction sites flanking the segment of DNA to be deleted, digesting with *Nhe*I, and then ligating the new ends back together. All mutant constructs were sequenced to verify mutagenesis. Supercoiled plasmid DNA expressing various constructs were prepared by double banding in a cesium chloride gradient (32).

Cell Culture & Microinjection

Mouse 10T1/2 fibroblasts were plated in Dulbeco's Modification of Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS). The cells were maintained at 37°C, 10% CO_2 and split just before reaching confluence. For microinjection, cells were plated onto glass cover slips and grown to 70% confluence in DMEM + 10% FBS. The cover slips were then transferred to DMEM containing 0.05% calf serum. After twenty-four hours of incubation in the FBS-free media, 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 μg/ml expression plasmid DNA and 6 mg/ml of either guinea pig or rabbit IgG (Sigma). All microinjection experiments were performed using an automatic micromanipulator (Eppendorf), with glass needles pulled on a vertical pipette puller (Kopf).

Immunostaining

For detection of Ret/ptc2 protein, cells were fixed in 3.7% formaldehyde five hours after injection for five minutes, and then washed with phosphate buffered saline (PBS). The cells were then incubated successively with rabbit anti-Ret (dilution 1:500), biotinylated donkey anti-rabbit IgG (dilution 1:400, Jackson ImmunoResearch), Texas Red streptavidin (dilution 1:100, Amersham), and FITC anti-guinea pig IgG (dilution 1:100, Jackson).

DNA synthesis was assessed by incorporation of the thymidine analog 5-bromodeoxyuridine (BrdU) and its subsequent detection by immunostaining (33). Following nuclear microinjection, 0.1 % BrdU labeling reagent (Amersham) was added to the starvation medium (DMEM + 0.05% calf serum), and the cells were

incubated for an additional 24 hours. Cells were fixed in 95 % ethanol/5 % acetic acid for thirty minutes, and then washed with PBS. Incorporation of BrdU was visualized by successively incubating the fixed cells with mouse anti-BrdU (undiluted, Amersham), biotinylated donkey anti-mouse IgG (dilution 1:500, Jackson), Texas Red streptavidin (dilution 1:100, Amersham), and FITC anti-rabbit IgG (dilution 1:100, Jackson).

Two Hybrid Screen

A yeast two-hybrid screen was performed by the methods of Vojtek, et al (34), with reagents from Stan Hollenberg (Fred Hutchinson Cancer Research Center). Ret/ptc2 cDNA was subcloned into the LexA-fusion vector pBTM116, and coexpressed in the L40 strain of *S. cerevisiae* with an embryonic mouse random-primed cDNA library. From approximately two million co-transformants, 37 survived nutritional selection. Library plasmids were rescued from these colonies, and co-transformed with other LexA-fussions to test for Ret/ptc2 specific interaction. Seventeen of the library plasmids yielded co-transformants which were β-galactosidase positive with Ret/ptc2 as assessed by 5-bromo-4-chloro-3-indolyl-β-D-galactoside (IPTG) selection. Ten of these seventeen plasmids expressed proteins which also interacted with a LexA-RI fusion, indicating that they were not binding to the Ret portion of Ret/ptc2. The cDNA inserts of the remaining seven were sequenced by the dideoxy method (30), and sequences obtained were compared to the contents of Genebank using the BLAST program through the NIH/NCBI server on the World Wide Web.

β-Galactosidase Assay

Two hybrid transformants were assayed for β-galactosidase activity by solution assay (32). Single colonies were grown overnight in selective media at 30 °C. Aliquots from these cultures were then used to seed fresh cultures which were grown to an OD_{600} of approximately 0.5. Cell pellets from 5 ml cultures were resuspended in 0.5 ml of Z buffer (60 mM Na_2HPO_4, 40 mM Na_2HPO_4, 10 mM KCl, 1 mM $MgSO_4$, 50 mM β-mercaptoethanol, pH 7.0). Samples of these resuspensions were diluted 5 to 20 fold (1 ml final volume) in Z buffer. Cells were lysed by addition of SDS and chloroform followed by vortexing. The chromogenic substrate o-nitrophenyl-β-D-galactoside was added (200 µl of 4 mg/ml solution), and the reaction was quenched by addition of 0.5 ml of 1 M Na_2CO_3. Units of activity are were calculated as: Activity = $1750(OD_{420})/[(\text{time in min})(\text{volume of culture in assay})(OD_{600} \text{ of culture})]$.

GST-Fusion Affinity Precipitation

Yeast two-hybrid results were verified using a stably transfected NIH3T3 cell line expressing an EGFR/Ret chimeric protein (35). These cells were treated with 100 nM EGF for 10 minutes 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 X-100, 1 mM benzamidine, 1 mM TPCK, 1 mM TLCK, 1 mM PMSF, 1 mM $NaVO_4$). Cleared lysates were incubated for 2 hours with 2 µg of GST fusion protein bound to glutathione agarose beads in a total volume of 300 µl. The beads were washed four

times with lysis buffer, resuspended in SDS-PAGE sample buffer, boiled, and electrophoresed in a 7% polyacrylamide get. Proteins were transferred to PVDF membranes and probed with either rabbit anti-Ret (36) (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-PLCγ - murine PLCγ SH2 domain 1 obtained from the two-hybrid screen; GST-Grb10 - murine Grb10 SH2 domain obtained from the two-hybrid screen.

His_6-Ret/ptc2 Expression and Purification

The *ret/ptc2* gene was subcloned into the bacterial expression vector, pET15b (Novagen), and expressed as a sexta-histidine fusion protein in the *E. coli* strain, NovaBlue(DE3), containing the *dnaY* gene. The *dnaY* gene product is necessary for providing tRNAs which decode arginine codons that predominate mammalian genes which are rarely found in bacterial hosts (37). Cells were grown in M9ZB medium containing 35 mg/l kanamycin and 100 mg/l ampicillin at 37 °C in a 5 l fermentor (New Brunswick) to an optical density of 1.2 at 600 nm. Protein expression was induced with 1 mM IPTG at 30 °C for 3 hours. Cells were harvested by centrifugation and stored at -80 °C until used. Frozen pellets from 10 l of cells were thawed on ice and lysed in a cold French pressure cell in the following buffer: 50 mM HEPES, pH 8, 150 mM NaCl, 10 % glycerol, 5 mM β-mercaptoethanol, 1 μg/ml leupeptin, 100 μg/ml TPCK, 50 μg/ml TLCK, 1 mM PMSF, 0.1 mM 1, 10-o-phenanthroline, 50 mM imidazole. His_6-Ret/ptc2 was partially purified from the crude supernatant fraction by affinity chromatography to Ni^+-NTA resin (Invitrogen), and to approximately 80 % homogenity in 50 mM HEPES, pH 8, 150 mM NaCl, 10 % glycerol, 5 mM β-mercaptoethanol by FPLC using a Superdex 200 gel filtration column. Peak fractions were collected and analyzed by Western blots using a polyclonal rabbit Ret antibody made against residues 535-551 conjugated to keyhole limpet haemocyanin (Calbiochem).

Ret/ptc2 Expression in Mammalian Cell Culture

Human kidney 293 cells were cultured at 37 °C, 8 % CO_2 on 10 cm dishes (Nunc) in DMEM containing L-glutamine and 1.0 g glucose/l supplemented with 5 % FBS, 5 % enriched calf serum, and 1 % fungibact. Cells were plated onto 10 cm dishes at 0.5 x 10^6 and incubated for at least 24 hours at 37 °C, 8 % CO_2 prior to transfection. Plasmid DNA was prepared from overnight cultures using an endotoxin-free maxi-prep kit following the manufacturer's protocol (Qiagen) and resuspended in 10 mM Tris-HCl, 1 mM EDTA, pH 8. 0.5 ml 0.25 M $CaCl_2$ and 2x 50 mM BES (N,N-bis[2-hydroxyl]-2-aminoethanesulfonic acid), 280 mM NaCl, 1.5 mM Na_2HPO_4-$2H_2O$ were added to 20 μg of endo-free plasmid DNA and mixed thoroughly. The mixtures were allowed to incubate at room temperature for at least 20 minutes, before adding each DNA mixture dropwise to a dish of cells along one edge while gently swirling the medium. Cells used for negative controls were transfected with an equivalent volume of endo-free TE, pH 8. All transfected cells

were incubated overnight at 37 °C, 3 % CO_2. The medium was aspirated off the cells the following day, cells were rinsed gently with 5 mls fresh medium, and incubated at 37 °C, 8 % CO_2 with 10 mls fresh medium for at least 48 hours before harvesting.

Cells were resuspended with 10 mls PBS and pelleted. Cell pellets were rinsed twice with 1 ml PBS and resuspended in 50 µl ice-cold lysis buffer: 50 mM HEPES, pH 7.95, 150 mM NaCl, 10 % glycerol, 5 mM β-ME, 1 µg/ml leupeptin, 100 µg/ml TPCK, 50 µg/ml TLCK, 1 mM PMSF, 0.1 mM 1,10-o-phenanthroline. Cells were lysed by homogenization on ice and the final volume was adjusted to 100 µl lysis buffer/10 cm dish. Cell lysates were centrifuged at 13,000 x g for 15 minutes at 4 °C. The supernatant fraction (S1) was withdrawn into cold microfuge tubes and stored on ice until used.

Kinase Assay

The S1 fraction was added to microfuge tubes on ice containing 1 mM peptide substrate in a final concentration of 50 mM HEPES, pH 7.95, 150 mM NaCl. The reactions were initiated at room temperature by adding 10 µl of a mixture containing 50 mM HEPES, pH 7.95, 200 µM $NaV0_4$, 40 mM $MnCl_2$, ATP, [γ-^{32}P]-ATP, briefly vortexed to mix, and incubated at room temperature for 10 minutes. The reactions were quenched with 17 µl ice-cold 24 % trichloroacetic acid, vortexed briefly, and returned to the ice. Ten microliters of ice-cold 10 mg/ml BSA was added to each sample followed by a brief vortex. Samples incubated on ice for 10 minutes, and then were centrifuged at 12,000 x g for 5 minutes. Fifty microliters from each sample was spotted onto P81 filter disks (Whatman). The disks were washed in 400-500 mls 75 mM H_3PO_4 for 2-5 minute intervals and 2-10 minute intervals at room temperature and 100 rpm. The disks were rinsed briefly in acetone, dried under a heat lamp, and quantitated by liquid scintillation counting in 5 mls Ecolume (ICN). The angiotensin II peptide was purchased from Sigma. All other peptide substrates were synthesized in the laboratory of Dr. E. Komives, Department of Chemistry and Biochemistry, UC San Diego.

The ATP Km for Ret/ptc2, the epidermal growth factor receptor (EGFR) chimera, and the EGFR kinase domain expressed in 293 cells was determined using 1 mM peptide substrate and varying the ATP concentration. ATP concentration vs. µmoles ATP incorporated was plotted and analyzed by KaleidaGraph.

RESULTS AND DISCUSSION

Modeling of the Ret Tyrosine Kinase Domain

Our modeling has served as a framework for understanding the structure of this protein and it is becoming even more important for our thinking about how the various additional proteins dock with the kinase domain. A particular focus now is on the C-terminal tail. Although this lies beyond the conserved catalytic core that is shared by all members of the protein kinase family, the C-terminus of Ret bears remarkable similarities to the C-terminus of the cAPK catalytic subunit. Clearly, a very long-term goal will be to obtain a structure of the kinase domain bound to Enigma or to one of the src-homology domains (SH2) of proteins identified in the yeast two-hybrid screen.

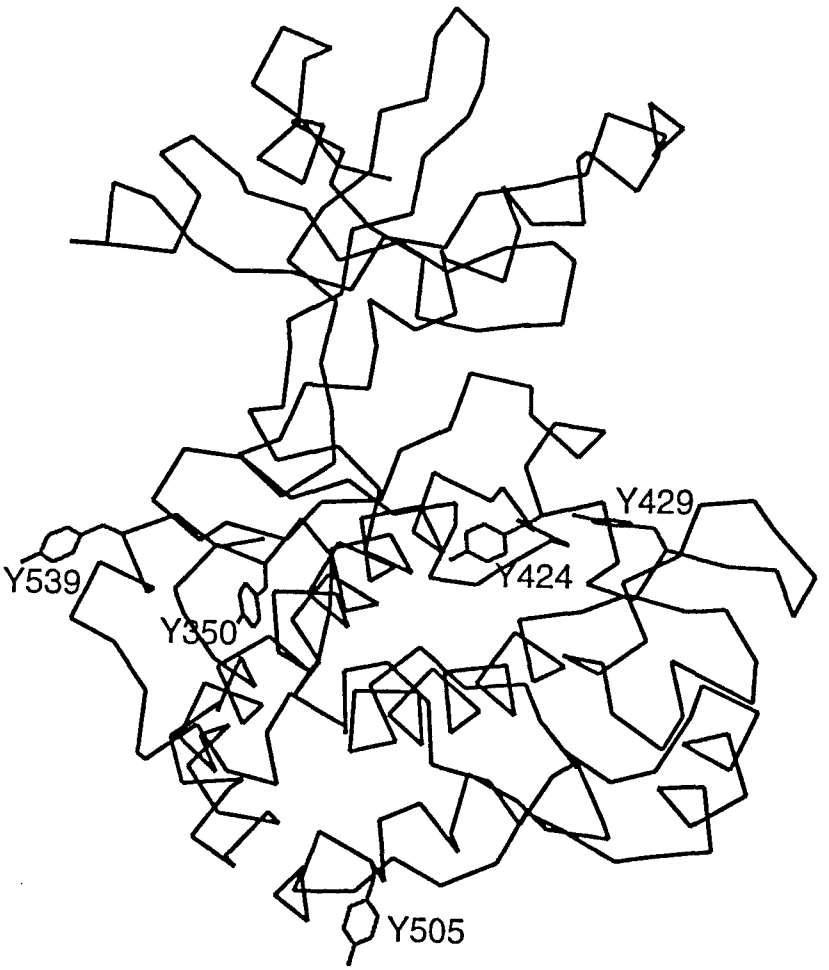

Figure 2. Computer model of the Ret/ptc2 tyrosine kinase domain from residues 237-542. The identified tyrosine residues have been shown to be important in eliciting a mitogenic response in the microinjection assay. This model does not include tyrosine 586 which is located at the C-terminus.

Development of the Mitogenic Assay

To evaluate which structural features of Ret/ptc2 are essential for its mitogenic activity, an expression plasmid microinjection assay was developed. This assay allows for both rapid screening of protein expression and evaluation of mitogenic activity. The expression vector, pRet/ptc2, was co-injected into nuclei of serum-starved 10T1/2 fibroblasts with rabbit or guinea pig IgG, which served as an injection marker. After incubation for 24 hours in 5-bromodeoxyuridine (BrdU) containing starvation media, the cells were fixed and stained for the IgG injection marker and either for Ret/ptc2 protein expression or DNA synthesis as assessed by BrdU incorporation. An identical construct containing the chloramphenicol acetyl transferase gene (pCAT) was used as a negative control.

Cells injected with pRet/ptc2 expressed protein detectable by anti-Ret antibodies as early as 5 hours post-injection. The expressed protein was cytoplasmic, and the amount and distribution of expressed protein was indistinguishable from that observed in pRet/ptc2 injected cells for all of the mutants discussed in this report. A plasmid concentration of 100 μg/ml was chosen for injections. Under these conditions, over 30% of the pRet/ptc2 injected cells entered S-phase compared to less than 6% of the pCAT injected cells (Figure 3).

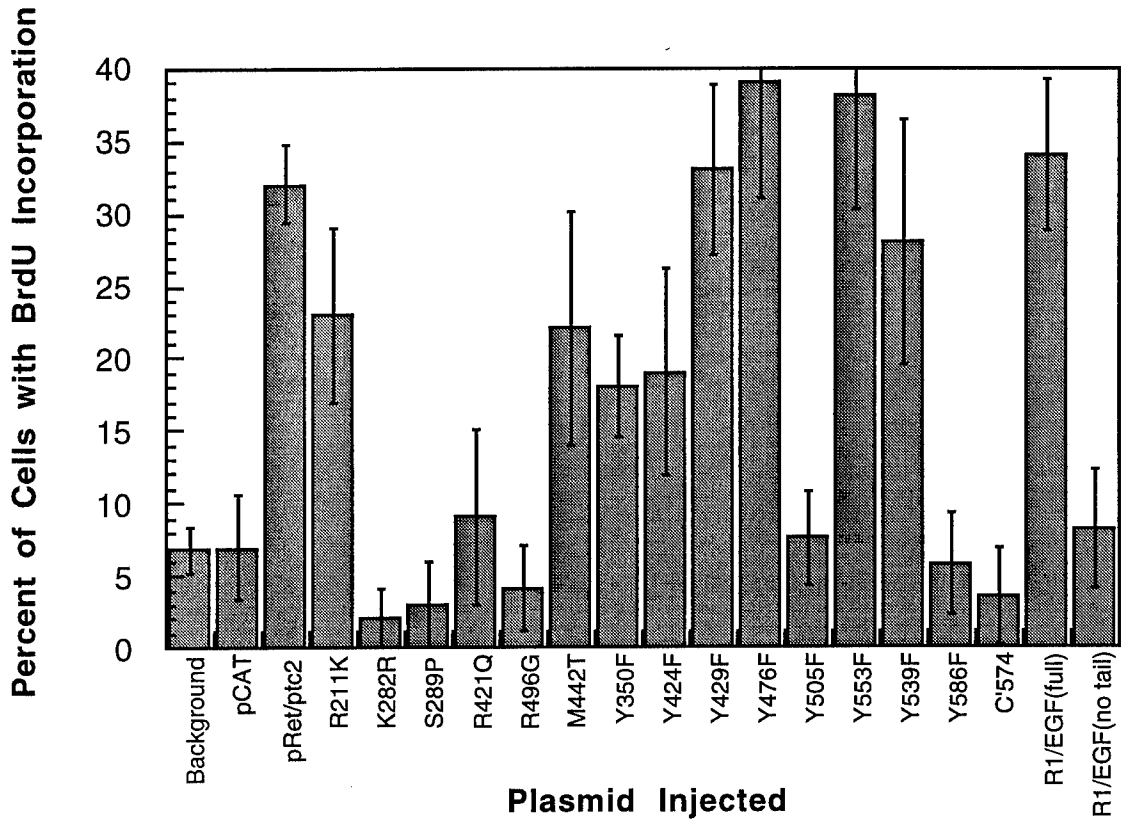

Figure 3. Mitogenic response of various constructs in the microinjection assay.

Consequences of Mutations in Ret/ptc2

Using this assay, the mitogenic activity of various Ret/ptc2 point mutants was tested (Figure 3). To determine whether cAMP binding was important for Ret/ptc2 activity, Arg 211 was mutated to Lys. This change eliminates high affinity cAMP binding in the A binding site of RIα (32); however, this point mutation had no significant effect on Ret/ptc2 mitogenic activity. In contrast, elimination of a conserved Lys in the kinase domain (38), K282R, eliminated *in vivo* mitogenic activity. Likewise, the three reported Hirschsprung's disease point mutations located in the Proto-ret tyrosine kinase domain, S289P, R421Q, and R496G, all inactivated Ret/ptc2 in this assay. These results support the model that

Hirschsprung's disease results from a loss of Proto-ret function and are consistent with recently obtained results using transfection assays on NIH3T3 and PC12 cell lines (39). The MEN2B mutation (M442T) had no effect on this activated form of Proto-ret, which does not contradict a recent report that this mutation alters substrate specificity rather than catalytic activity of Proto-ret (38).

Role of RIα in Ret/ptc2

The N-terminus of Ret/ptc2 is comprised of the first 236 residues of RIα which includes the RIα dimerization domain, the PKA auto-inhibitory site, and most of RIα's first cAMP binding domain (2). This is fused to the cytoplasmic portion of the Ret receptor which consists of its tyrosine kinase domain (Ret_{tk}) followed by a short C-terminal tail. The wild-type RIα subunit is a disulfide-bonded dimer with the protomers aligned through residue 37 in an antiparallel orientation (26). Constructs expressing deletion mutants were tested to determine the role of RIα in the activation of Ret_{tk} (Figure 4). These mutants showed clearly that the only portion of RIα required for Ret/ptc2 mitogenic activity was the dimerization domain (Figure 4) and support the hypothesis that the Ret_{tk} is activated in Ret/ptc2 via the dimerization domain of RIα.

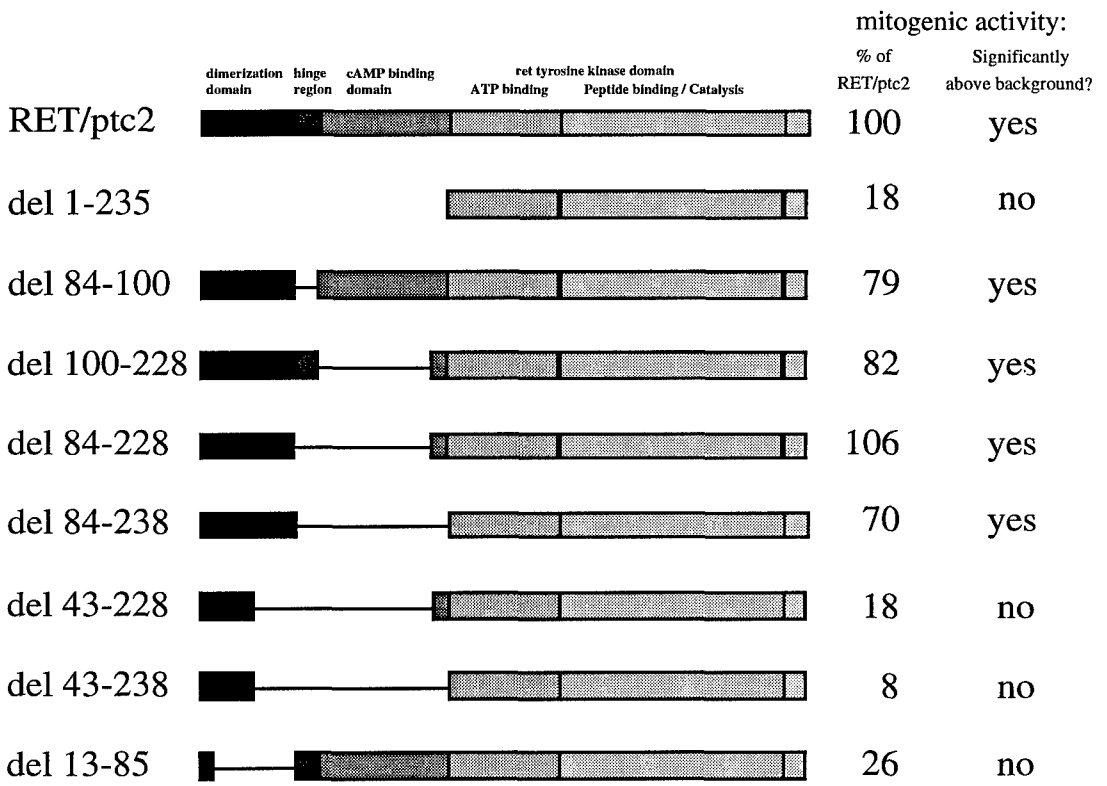

Figure 4. Mitogenic response of various Ret/ptc2 deletion constructs in the microinjection assay.

To investigate whether RIα mediated dimerization is a general activating motif for receptor tyrosine kinases, an analogous construct to Ret/ptc2 was made by substituting the epidermal growth factor receptor tyrosine kinase (EGF_{tk}) for the Ret_{tk}. The expression construct encoding RIα residues 1 to 236 fused to the EGF_{tk} and its C-terminal tail, residues 647 to 1186, was as active as pRet/ptc2 in our microinjection assay (Figure 3). The carboxyl terminal tail, residues 959 to 1186, contains all of the known SH2 docking sites of the EGF receptor (40). Deletion of this tail in the $RI\alpha/EGF_{tk}$ construct eliminated its mitogenic response in our assay (Figure 3). C-terminal truncations of the EGFR have been shown to have increased transforming activity attributed to an inability to internalize and attenuate the EGF signal (41, 42). The complete loss of mitogenic activity for our C-terminal EGF_{tk} deletion suggests that SH2 domain interactions are more important for the mitogenic response of $RI\alpha/EGF_{tk}$ than for C-terminal truncations of the EGF receptor.

Role of Tyrosines in Ret/ptc2

To identify possible SH2 interaction sites in Ret/ptc2, several tyrosines were mutated to phenylalanine and the resultant mutants tested for mitogenic activity (Figure 3). Of the eight tyrosine mutants, Y350F and Y424F exhibited significantly reduced mitogenic activity. Mutations at two other tyrosines, Y505F and Y586F, abolished the mitogenic effect. Y350 is located in a variable insert region of receptor tyrosine kinases, and in the PDGF receptor this insert region contains two SH2 binding sites (43). Y424 in Ret/ptc2 corresponds to Y1158 in the insulin receptor (43), and phosphorylation at Y1158 is required for full activity. Y505 is in a sequence which aligns with the H-helix of the insulin receptor tyrosine kinase (29, 38). This tyrosine is highly conserved in the receptor tyrosine kinases (43), and could serve a structural role rather than as an SH2 docking site. Tyrosine 586, which completely eliminated mitogenic activity when mutated to Phe, is in the C-terminal tail of the Ret_{tk}.

Tyrosines 586 and 350 are the best candidates for residues that provide SH2 or PTB docking sites essential for the mitogenic response of Ret/ptc2. Mutation of the other tyrosine in the C-terminal tail, Y553, had no effect on mitogenic activity.

Yeast Two-Hybrid Interactions

To search for proteins that interact with the Ret/ptc2 oncogene, a mouse random-primed cDNA library was screened using a yeast two-hybrid system (34). Three vectors isolated from the library by interaction with Ret/ptc2 encoded the following mouse sequences: the C-terminal 155 residues of Grb10 (44); 156 residues which share 97% identity with residues 537-693 of rat PLCγ2 (45); and 131 residues with 95% identity to the C-terminal 131 residues of human Enigma, containing all of LIM2 and 3 (46). These sequences corresponded to the SH2 domain of Grb10, the first SH2 domain of phospholipase-C-γ (PLCγ), and a C-terminal fragment of Enigma that contained LIM domains 2 and 3 (LIM2/3). The interaction of Ret with PLCγ and Grb10 has been observed previously (35, 47). Using the two-hybrid system, the interactions of these proteins with Ret/ptc2 were characterized.

Mutants of Ret/ptc2 were subcloned into the two-hybrid vector and interactions were quantitated by β-galactosidase activity (Figure 5). 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 tyrosine to phenylalanine mutants of Ret/ptc2, residues required for Grb10 and PLCγ binding were identified as Y429 and Y539, 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 when the C-terminal 23 residues of Ret/ptc2 were deleted (C'574).

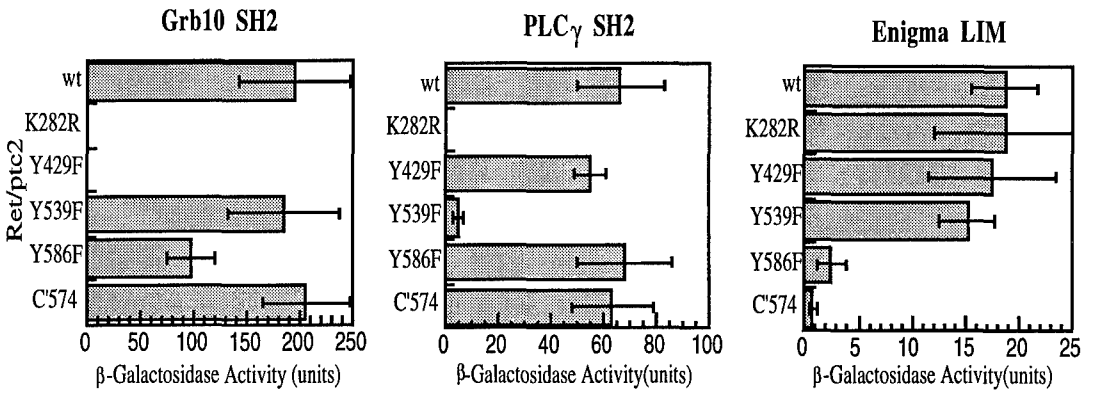

Figure 5. β-galactosidase activity of yeast co-transfected with constructs expressing Ret/ptc2 as bait (wild-type or tyrosine to phenylalanine mutants) and with Grb10-SH2, PLCγ-SH2, or Enigma-LIMs as prey.

In Vitro Binding to EGFR/Ret

To verify the yeast two-hybrid results, affinity precipitation experiments were performed. The binding domains from PLCγ, Grb10, and Enigma were expressed in *E. coli* as glutathione-S-transferase (GST)-fusion proteins. The three GST-fusion proteins were incubated with lysates of NIH3T3 cells expressing the EGFR/Ret chimeric receptor, where EGF-dependent activation of EGFR/Ret has been characterized (35). In each case binding to EGFR/Ret isolated from lysates of EGF-treated cells was observed. 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 the PLCγ, Grb10 and v-Src GST-SH2 domains required EGF-stimulated receptor auto-phosphorylation. 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 (21). The long form binds to Grb2 (48) 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 (35).

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 (49, 50), where higher expression levels were achieved, it was possible to observe the phosphorylation state of Ret using anti-phosphotyrosine antibodies. Enigma bound to both Ret/ptc2 and to the Ret tyrosine kinase alone (51), but did not bind to either when the C-terminal 23 residues were deleted. The interaction was not dependent upon the phosphorylation state of Ret because Ret_{tk} was not phosphorylated on tyrosine in the absence of the dimerization domain of RIα, whereas Ret/ptc2 underwent tyrosine autophosphorylation.

Based on optical densitometry, the extent of tyrosine phosphorylation of Ret/ptc2 and the C-terminal deletion Ret/ptc2 C'574 were equivalent. Because deletion of the C-terminus of Ret/ptc2 did not decrease the phosphotyrosine content by a detectable amount, Y586, the only tyrosine in the C-terminus of this isoform of Ret, does not appear to be a major site of autophosphorylation. Results using both the EGFR/Ret chimera and Ret/ptc2 thus indicated that interaction with Enigma required the C-terminus containing Y586 but was independent of tyrosine autophosphorylation of Ret. The interaction between LIM2 of Enigma and Ret was specific because LIM domains from other proteins such as zyxin and the cysteine-rich protein failed to interact with Ret/ptc2 (52). Another receptor tyrosine kinase, EGFR, failed to interact with the LIM domains of Enigma either in the two hybrid system or in GST-fusion binding reactions.

Effect of Enigma on Ret/ptc2 Mitogenic Activity

The functional significance of the association between Ret and Enigma was investigated *in vivo* using the microinjection assay. As shown in Figure 3, mutations in Ret/ptc2 that blocked association with Grb10 (Y429F) or PLCγ (Y539F) had no significant effect on mitogenic activity, while mutation of tyrosine 586 to phenylalanine or deletion of the C-terminus of Ret/ptc2 completely blocked the ability of Ret/ptc2 to induce DNA synthesis.

The strong correlation between mutants which failed to bind Enigma and loss of mitogenic activity suggested that Enigma was either required for the mitogenic signaling of Ret/ptc2 or that some other protein, which was not detected in the two-hybrid screen, also binds at Y586. To discriminate between these two possibilities, co-injection experiments were performed to attempt to block the Ret/ptc2 mitogenic signal. Co-injection of Ret/ptc2 with a plasmid which expressed the three LIM domains of Enigma (C275, Figure 6) blocked Ret-induced DNA synthesis, while co-injection with full-length Enigma had no effect. These results support the conclusion that Ret/ptc2 requires Enigma for mitogenic signaling. If Enigma was simply competing for binding with some other signaling protein, then both full-

length Enigma and the LIM domains alone should block signaling, given that both interact with Ret/ptc2 with equivalent affinity (51). The inhibition of mitogenesis was specific to the LIM domains of Enigma because the LIM domains of zyxin (53) were without effect. It was also specific for Ret because the LIM domains of Enigma did not block the mitogenic activity of the EGFR tyrosine kinase analog of Ret/ptc2 (RIα/EGFR) shown to have mitogenic activity in this assay (Figure 3)

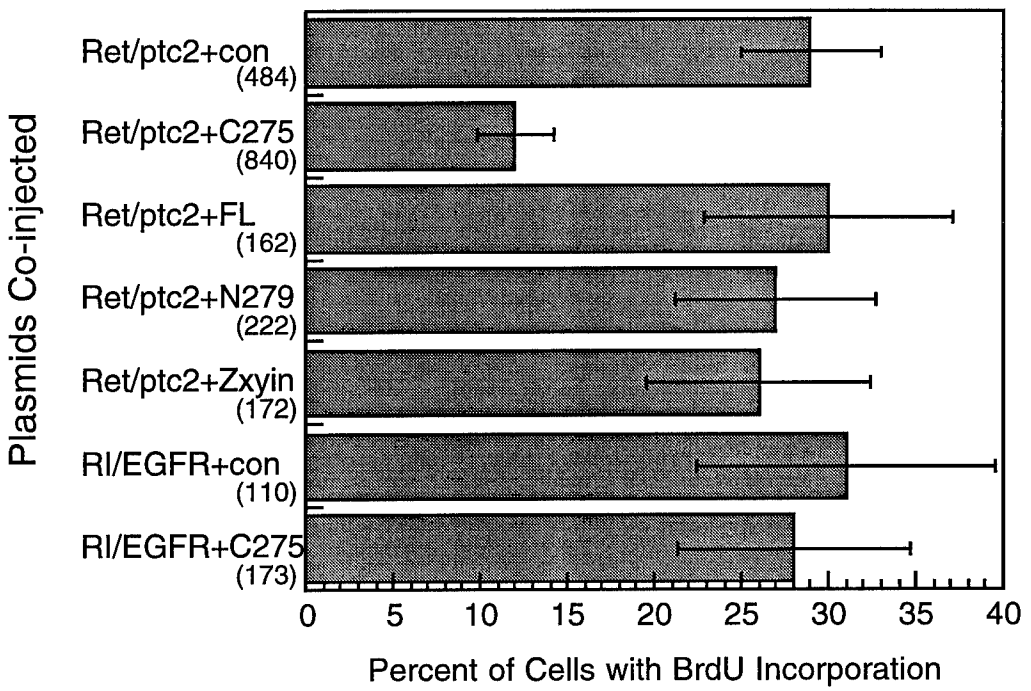

Figure 6. Mitogenic response of Ret/ptc2 co-injected with the control expression plasmid (con), full-length Enigma (FL), C-terminal half of Enigma (C275), or N-terminal half of Enigma (N279).

Proto-ret Interaction with PTP1C

In a recent collaboration with the lab of Jack Dixon (U. Michigan, Ann Arbor), we have characterized the interaction between Ret/ptc2 and Protein Tyrosine Phosphatase 1C (Pandey, *et al.*, submitted). PTP1C contains two SH2 domains at its N-terminus, and both are capable of binding to Proto-ret. In co-transfection experiments, PTP1C was phosphorylated at a single site by Ret/ptc2. This site, tyrosine 538, is a potential Grb2-SH2 interaction site, but no recruitment of Grb2 to a Ret/ptc2-PTP1C complex was observed. In addition, co-injection of Ret/ptc2 expression constructs with constructs expressing either a phosphatase-dead or

tyrosine 538 to Phe PTP1C had no effect on mitogenic activity. We've concluded that PTP1C is not important for the mitogenic activity of Ret/ptc2, but it may be required for the non-mitogenic functions of Proto-ret.

Interaction of Ret/ptc2 with Shc

It has recently been reported that the adaptor protein Shc binds to Proto-ret at tyrosine 1162 (M. Pierotti, submitted). This site corresponds to tyrosine 586 of Ret/ptc2, the site we have shown interacts with Enigma. We are currently mapping the amino acid determinants for these two interactions in hopes of creating mutants of Ret/ptc2 which will only interact with Enigma or Shc but not both. In addition, we are expressing full-length and the binding domains of Shc as GST-fusion proteins in *E. coli*, as we have previously done with Enigma and its LIM domains(51). These reagents will allow us to further investigate the interactions *in vitro* .

Expression, Purification and Physical Characterization of His_6Ret/ptc2

The expression of Ret/ptc2 in *E. coli* has proved to be extremely difficult and has taken much more time than we anticipated. We have been able to vary the conditions to optimize the expression Ret/ptc2, however, we still do not have sufficient quantities for crystallization. The final yield of His_6-Ret/ptc2 from 10 l of bacterial cells is approximately 2-3 mg. The isoelectric point of His_6-Ret/ptc2 is approximately 4.6. By calibrating the FPLC Superdex 200 column with protein standards (Pharmacia), the stokes radius of His_6-Ret/ptc2 was determined to be 51 Å which corresponds to a molecular weight of 185 kDa. These results indicate that: 1) His_6-Ret/ptc2 maintains an elongated shape in solution which is consistent with the antiparallel, helical structure of the R-subunit dimerization domain (26, 54), and 2) this verifies that the active form of Ret/ptc2 is a dimer. The dimerization domain of Ret/ptc2 was shown to be critical for eliciting a mitogenic response in microinjection studies using mouse 10 T 1/2 cells since deletion of this domain abrogated the mitogenic response (36). Further characterization of the hydrodynamic properties of His_6-Ret/ptc2 will include determination of the sedimentation and frictional coefficients.

Peptide Substrate Screening and Selection

In a modified phosphocellulose kinase assay (55), His_6-Ret/ptc2 phosphorylates the angiotensin II peptide substrate but neither the EGFR C-terminal 1173 peptide nor the Src peptide is phosphorylated.

Since His_6-Ret/ptc2 autophosphorylates *in vivo*, it was clear that a suitable peptide substrate could be derived from internal sequences containing tyrosine residues. A number of peptide substrates surrounding internal tyrosine residues identified as potential autophosphorylation sites from both the microinjection assay (36) and yeast two-hybrid screen were synthesized and tested at 1 mM in the kinase assay in the presence of 150 μM ATP and [γ-^{32}P]-ATP. Since Ret/ptc2 is autophosphorylated when expressed transiently in human kidney 293 cells, the crude supernatant fraction from lysed cells was used as a source of active enzyme for screening peptide substrates. The results are summarized in Table I.

TABLE I. Peptide Substrate Screen

Peptide Substrate	Sequence	μmoles Pi incorporated min^{-1}
angiotensin II	DRVYHPF	2.1
35b	RRKSRDVY$_{424}$EEDS-NH_2	0.089
36b	RRKSEEMY$_{505}$RLML-NH_2	0.21
37	KRRDY$_{539}$LDLA	1.6
21	KRRDY$_{539}$LDLAASTPSDSL	0.83
39	RKVGPGY$_{350}$LGSG-NH_2	0.01
42	KRRENKLY$_{586}$GRIS-NH_2	0.42
43	KRRSRDVY$_{424}$EEDSY$_{429}$VKRS-NH_2	0.53
44	RPAQAFPVSY$_{687}$SSSG	0
45	KRREEDSY$_{429}$VKRS-NH_2	0.11
49	AEEEEYFELVAKKKK	0.01

Although the angiotensin peptide appears to be a better *in vitro* substrate for Ret/ptc2, the results of the assay demonstrated that peptide 37 is a more specific substrate. Peptide substrate 37 contains Y539 which was found in the yeast 2-hybrid screen to be an interaction site for PLC-γ. Peptide 37 is a shorter version of peptide 21, however the degree of phosphorylation of peptide 37 by Ret/ptc2 is significantly increased 2-fold.

Peptides 35b and 45 contain Y424 and Y429, respectively, of Ret/ptc2 which correspond to Y1158 and Y1162 in the insulin receptor (29). Phosphorylation of these residues in the insulin receptor is critical for activity. Phosphorylation of either tyrosine residue in peptide substrate 35b or 45 is minimal, however, when both residues are present within a single peptide, as in peptide 43, there appears to be a synergistic effect.

Microinjection studies indicated that mutation of either Y505 or Y586 to phenylalanine significantly decreased the mitogenic response (36). Using a yeast two-hybrid screen, we found that Y586 interacts with a LIM domain containing protein, Enigma, however the interaction did not depend on the phosphorylation of this residue (36, 51). Recently, phosphorylated Y586 has been proposed to be an interaction site for Shc (M Pierotti, manuscript submitted). In the kinase assay,

phosphorylation of Y505 in peptide 36b was marginal whereas phosphorylation of Y586 in peptide 42 by Ret/ptc2 was moderate.

Mutation of Y350 to phenylalanine decreased the mitogenic response by 30 % (36), however, peptide 39 was poorly phosphorylated by Ret/ptc2 *in vitro*. Peptide 44 corresponds to the juxtamembrane sequence of Proto-ret. Both Y350 and Y687 were identified to be autophosphorylated by 2-dimensional phosphopeptide mapping of immunoprecipitated Proto-ret isolated from transiently transfected COS cells with *proto-ret* and subsequently autophosphorylated *in vitro* with [γ-^{32}P]-ATP (56). Peptide 49 is a consensus peptide substrate sequence for the EGFR (57).

The ATP Km was determined to be 51-61 μM ATP for Ret/ptc2 expressed in 293 cells. The following plot (Figure 7) is a representative example of the kinase assay data. Each point is the average of 3 data points, the standard errors for the data points is 9 % or less, and the correlation factor is 0.997.

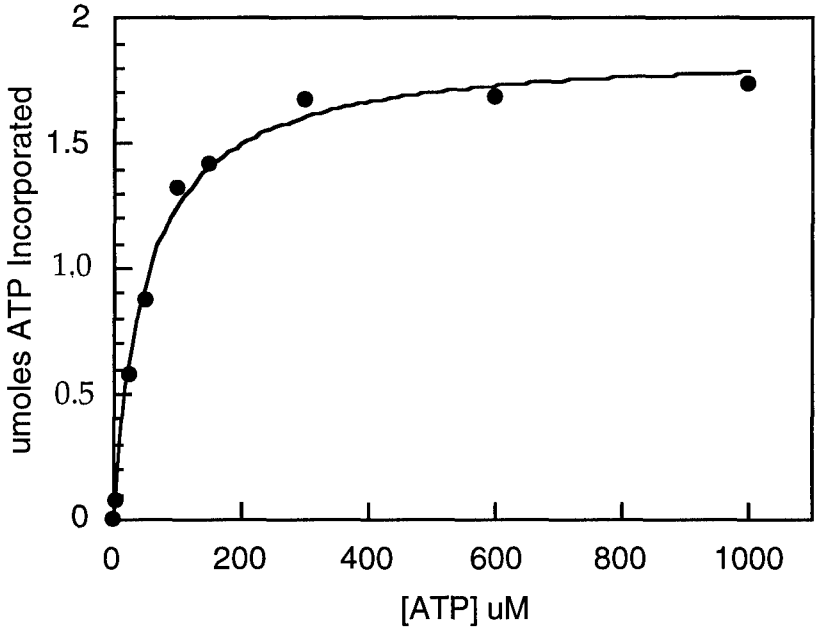

Figure 7. ATP Km for Ret/ptc2 expressed in human kidney 293 cells.

Analysis of the Epidermal Growth Factor Receptor and Insulin Receptor Chimeric Proteins

The generally accepted dogma of receptor tyrosine kinase (RTK) activation is that upon ligand binding monomers dimerize resulting in a conformational change within their intracellular kinase domains leading to autophosphorylation which elicits downstream signaling events (58). This model seems logical when the proteins are embedded in the membrane and one can envision autophosphorylation of the parallel dimers by either an intra- or intermolecular

process. However, the activation of a protein kinase in an antiparallel arrangement presents an intriguing paradox.

We have found the RIα dimerization domain is an absolute requirement for Ret/ptc2 activity (36). Activation of the Ret/ptc2 dimer can occur if it has a flexible hinge region which allows the C-terminal tail to loop around and autophosphorylate either intra- or intermolecularly at a distal critical tyrosine residue. Alternatively, dimers can form oligomeric chains such that phosphorylation occurs as a intermolecular process.

In order to investigate the role of the RIα dimerization domain in the activation of the tyrosine kinase domains of RTKs, we constructed chimeric genes of both the epidermal growth factor receptor (EGFR) and the insulin receptor (InsR) to mimic the *ret/ptc2* gene. In the microinjection assay, the EGFR chimera elicited a mitogenic response similar to that of Ret/ptc2, however, removal of the C-terminal tail, residues 959-1186, abrogated this response (36). Similar to Ret/ptc2, the EGFR chimera is autophosphorylated when expressed in 293 cells and the protein can be phosphorylated *in vitro*. The ATP Km was determined to be 62-79 μM in the kinase assay using the EGFR 1173 peptide substrate at 1 mM. The ATP Km for the EGFR kinase domain is 48-57 μM. These results indicate that the contribution of the RIα subunit to the activity of the EGFR kinase activity is not significant *in vitro*. However, the strong mitogenic response elicited by the EGFR chimera suggests that the *in vivo* contribution of the dimerization domain is significant. We have recent evidence that the RIα subunit dimerization domain interacts with an anchoring protein, D-AKAP1 (Huang, J.-S. et al., 1996, manuscript submitted) which serves to localize RIα within the cytosol. In contrast to the EGFR chimera, expression of the InsR chimera in 293 cells resulted in an inactive, unphosphorylated protein. These results suggest that the activation of the Ret/ptc2 and EGFR kinase domains occurs via an intramolecular process whereas the InsR kinase may be activated by an intermolecular event. To investigate this further we are substituting the parallel dimerization domain of the GCN4 gene (59) for the antiparallel dimerization domain of the RIα subunit to test for both *in vivo* and *in vitro* activity.

CONCLUSIONS

This represents a new project for my laboratory, and our progress during the past two and a half years has been very good. We have made significant contributions towards an understanding of the *ret/ptc2* oncogene. Much of our work is still in progress, but our research has already led directly to three publications. An additional manuscript has been submitted and two others are in preparation. These are listed below, and reprints are included in the appendix. Our progress and ongoing work can be summarized in three general areas.

We have modeled the kinase portion of Ret based on our structure of the cAPK C-subunit and of the crystal structure of the insulin receptor kinase domain. This has provided a structural framework for the rest of our research. We are now focusing on a comprehensive model of all of the genetic lesions and are looking closely at the C-terminal tail for similarities to the catalytic subunit of cAPK.

With the biological assay we have been able to establish what is essential for the mitogenic properties of *ret/ptc2*. In conjunction with this, we have used the yeast two-hybrid system to identify Ret/ptc2 binding proteins and are developing a comprehensive picture of this receptor tyrosine kinase and its *in vivo* partners. The completely surprising discovery that Enigma binds specifically to Ret/Ptc2 has introduced a potentially important and novel element to this signaling pathway. Resolving the physiological role of Enigma is now a high priority. With a set of SH2 binding proteins in hand that bind to various regions of Ret/ptc2 we are also now in a position to more precisely define these protein:protein interactions and to determine what effect binding has on the structure and function. We are also now characterizing the subcellular localization of Ret/ptc2 using confocal microscopy. If docking to specific sites is thought to be important, then we shall also mutate residues within the dimerization domain of RIα to see if we can isolate mutant forms of Ret/ptc2 that are still dimers but no longer mitogenic. In parallel with the microinjection studies we have developed a transient mammalian-cell expression system and an *E. coli* expression system that yields mg quantities of Ret/ptc2. Finally, we have used this expressed protein to evaluate peptide substrates, and to developed an *in vitro* kinase assay. We are now initiating expression of Ret/ptc2 in *Pichia* to see if our yields increase. We shall also explore coexpression with Enigma and the various SH2 domain proteins to determine whether any provide enhanced stability. Structural analysis of the C-terminal tail bound to the LIM domains of Enigma is a good approach for defining the specificity requirements for LIM2. Another ongoing goal is to search for physiological substrates of Ret/ptc2.

In the yeast two-hybrid screen we also identified several novel proteins that bind to the N-terminus of RIα. These proteins have now been cloned and expressed in *E. coli*. Localization of these docking proteins is being carried out in parallel with the localization of activated Ret/ptc2. Like Enigma, the physiological role of this novel family of proteins is still not clear.

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APPENDIX

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Specificity of LIM Domain Interactions with Receptor Tyrosine Kinases*

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LIM domains, Cys-rich motifs containing approximately 50 amino acids found in a variety of proteins, are proposed to direct protein·protein interactions. To identify structural targets recognized by LIM domains, we have utilized random peptide library selection, the yeast two-hybrid system, and glutathione *S*-transferase fusions. Enigma contains three LIM domains within its carboxyl terminus and LIM3 of Enigma specifically recognizes active but not mutant endocytic codes of the insulin receptor (InsR) (Wu, R. Y., and Gill, G. N. (1994) *J. Biol. Chem.* 269, 25085–25090). Interaction of two random peptide libraries with glutathione *S*-transferase-LIM3 of Enigma indicated specific binding to Gly-Pro-Hyd-Gly-Pro-Hyd-Tyr-Ala corresponding to the major endocytic code of InsR. Peptide competition demonstrated that both Pro and Tyr residues were required for specific interaction of InsR with Enigma. In contrast to LIM3 of Enigma binding to InsR, LIM2 of Enigma associated specifically with the receptor tyrosine kinase, Ret. Ret was specific for LIM2 of Enigma and did not bind other LIM domains tested. Mutational analysis indicated that the residues responsible for binding to Enigma were localized to the carboxyl-terminal 61 amino acids of Ret. A peptide corresponding to the carboxyl-terminal 20 amino acids of Ret dissociated Enigma and Ret complexes, while a mutant that changed Asn-Lys-Leu-Tyr in the peptide to Ala-Lys-Leu-Ala or a peptide corresponding to exon16 of InsR failed to disrupt the complexes, indicating the Asn-Lys-Leu-Tyr sequence of Ret is essential to the recognition motif for LIM2 of Enigma. We conclude that LIM domains of Enigma recognize tyrosine-containing motifs with specificity residing in both the LIM domains and in the target structures.

LIM domains contain about 50 amino acids and have a cysteine-rich sequence: $CX_2CX_{16-23}CX_2HX_2CX_2CX_2CX_{16-23}CX_2C$ (1). They bind two atoms of Zn^{2+} with coordination being S_4, S_3N_1, S_3O_1, or $S_2N_1O_1$ (2). The NMR structure of the carboxyl-terminal LIM domain of chicken cysteine-rich protein (CRP)[1] revealed that the two Zn^{2+} binding modules are located at the ends of a hydrophobic core composed of antiparallel β sheets (3). LIM domains were initially recognized in the primary sequences of the homeodomain proteins Lin 11 (4), Isl-1 (5), and Mec-3 (6) and have subsequently been identified in a variety of homeodomain proteins (7, 8), in cytoskeleton-associated proteins (9, 10), in LIM domain-only proteins (11–13), in protein kinases (14), and in proteins of undefined function (8).

Most LIM proteins contain more than one LIM domain. The sequence of an individual LIM domain is, in general, more closely related to the same LIM domain in analogous proteins from other species than to other LIM domains within the same protein (8). Although the NMR structure of LIM2 of CRP resembles the DNA binding domain of the GATA-1 transcription factor (3), most available evidence indicates that LIM domains function in protein·protein rather than protein·DNA interactions.

Two structural targets for LIM domains have been identified. Using gel overlay techniques, Schmeichel and Beckerle (15) found that the LIM domains of zyxin interacted with the LIM-only protein CRP. Specificity was evident from the observation that LIM1 but not LIM2 or LIM3 of zyxin-bound CRP. Feurstein, *et al.* (16) also found evidence for LIM·LIM interactions involving CRP but did not observe specificity for the LIM domain. The carboxyl-terminal LIM domain of the cytoplasmic protein Enigma was found to specifically interact with exon 16 of the insulin receptor (InsR) (17). Mutations in exon 16 that disrupted the major endocytic code and ligand-induced endocytosis of InsR (18) also disrupted interaction with Enigma. The endocytic code of InsR, like that of many receptors (19), consists of 4–6 amino acids that form a tyrosine-containing tight turn (20). A generalized tight-turn motif, which functioned in endocytosis of mutant EGFR (21), and which contained two copies of an Asn-Asn-Ala-Tyr-Phe motif interacted with a wider range of LIM domains, suggesting that specific Tyr-based tight turns would provide interaction targets for specific LIM domains. There is functional evidence for LIM domain interactions with a variety of transcription factors (22–24), suggesting that additional target specificities exist.

We recently found that in addition to InsR, Enigma specifically interacted with the receptor tyrosine kinase Ret (25). Given that Enigma, which contains three LIM domains at its carboxyl terminus,[2] was found to interact with two receptor tyrosine kinases, determining the molecular basis of this recognition became important. We found that LIM2 of Enigma

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[1] The abbreviations used are: CRP, cysteine-rich protein; InsR, insulin receptor; Ret, receptor tyrosine kinase; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; GST, glutathione *S*-transferase; HA, hemagglutinin; PTB, phosphotyrosine binding.

[2] The LIM domains of Enigma are numbered from amino- to carboxyl-terminal location in the protein. LIM3 equals LIM2 in Wu and Gill (17).

specifically recognized Ret whereas LIM3 of Enigma specifically recognized InsR. Detailed analysis of the target sites indicated both LIM2 and LIM3 recognize Tyr-containing motifs located outside the tyrosine kinase cores of Ret and InsR. Individual LIM domains thus have the ability to distinguish between Tyr-based motifs providing a mechanism for specificity in both the LIM domain and in the target. This is of special interest given the requirement of the target of LIM2 in Ret for mitogenic signaling and of the target of LIM3 in InsR for endocytosis.

EXPERIMENTAL PROCEDURES

Materials—The *Caenorhabditis elegans* Mec-3 cDNA was obtained from Dr. Serge Lichsteiner, University of California Berkeley, Berkeley, CA; the zyxin and CRP cDNAs were obtained from Dr. Mary C. Beckerle, University of Utah, Salt Lake City, Utah; the cDNAs of Isl-1 and Xlm-1 were obtained from Dr. Mark Montminy, The Salk Institute, La Jolla, CA; and the cDNA encoding the LIM domains of paxillin were obtained from Dr. Michael Brown, State University of New York, Syracuse, NY. The Ret/ptc2 clone was a gift from Dr. M. Pierotti, Institute Nationale Tumori, Milan, Italy. NIH3T3 cells overexpressing the EGFR/Ret chimera were a gift of Dr. Pier P. Di Fiore, European Institute of Oncology, Milan, Italy (26). Fluorescence-activated cell sorting employing the 528 monoclonal anti-EGFR antibody was used to isolate a subline expressing high levels of this chimeric receptor. The 12CA5 anti-HA antibody was purchased from BAbCO, Berkeley, CA; the anti-InsR antibody (27) was a gift of Dr. Lynn Seely, University of California San Diego, La Jolla, CA, and the anti-phosphotyrosine antibody PY-20 was from Transduction Laboratories (Lexington, KY). A rabbit polycloncal anti-Ret antibody was raised against the 20-amino acid peptide (Lys-Arg-Arg-Asp-Tyr-Leu-Asp-Leu-Ala-Ala-Ser-Thr-Pro-Ser-Asp-Ser-Ilu-Tyr-Asp) of the carboxyl terminus of the shorter splice isoform of Ret (28). The residue numbering in the present report follows that of Ret/ptc2. For comparison with residue numbering in c-Ret, see Durick *et al.* (29).

Synthesis and Purification of Peptides—Peptides were synthesized on an Advanced Chemtech MPS 350 at the Center for Molecular Genetics Peptide Synthesis Facility (University of California, San Diego) using Fmoc (*N*-(9-fluorenyl)methoxycarbonyl) chemistry. Peptides were dissolved in water and purified on a Sephadex G-15 column equilibrated in 0.12 M triethylammonium bicarbonate. Peak fractions (monitored at A_{274} or A_{280}) were dried in a speed vac (Savant), resuspended in water, and dried until the pH became neutral (total of four times). Peptides containing Trp were quantitated using A_{280} and an extinction coefficient of 5600 $M^{-1}cm^{-1}$. Tyr-containing peptides were quantitated using A_{274} and an extinction coefficient of 1400 $M^{-1}cm^{-1}$. The peptide lacking any absorptive amino acid was quantitated using amino acid analysis.

Mammalian Expression Vectors—The HA expression plasmid was constructed by subcloning a DNA fragment that encoded a Met initiation codon and an HA epitope tag into the mammalian expression vector pcDNA3 (Invitrogen) at the *Bam*HI and *Eco*RI sites of the polylinker region. cDNA clones corresponding to various regions of Enigma were excised from the pJG4-5 yeast expression vector using the restriction enzymes *Eco*RI and *Xho*I and fused in-frame to the HA epitope-tagged vector. The Ret/ptc2 and mutant Ret/ptc2 constructs in the RC/CMV expression plasmid (Invitrogen) were prepared as described elsewhere (29). All constructs were confirmed by sequencing (U. S. Biochemical Corp.)

Production of GST Fusion Proteins—Construction of plasmids expressing GST fusion proteins with the LIM domains of Enigma has been described (17). Two polymerase chain reaction primers were synthesized and used to isolate a DNA fragment encoding the carboxyl-terminal 61 amino acids (residues 536–596) of Ret. The polymerase chain reaction product bearing 5′ *Eco*RI and 3′ *Sal*I sites was cloned into the polylinker region pGEX-KG (Pharmacia Biotech Inc.). GST fusion proteins were prepared using standard procedures and the fusion proteins were immobilized on glutathione agarose beads (Sigma).

The Yeast Two-hybrid System—Reagents and procedures for the Lex-A based-yeast two-hybrid system were utilized (30). cDNAs coding wild type and mutant Ret/ptc2 were used to create LexA-Ret/ptc2 fusion proteins. cDNA fragments coding all the tested LIM domains were isolated by using *Pfu* polymerase and oligonucleotides that contained *Eco*RI and *Xho*I sites and fused in-frame to the pJG4-5 vector. Plasmids that direct the synthesis of LIM domains and full-length Enigma were introduced into EGY48/1840 yeast that contained different LexA fusion Ret/ptc2 constructs using the lithium acetate procedure. All constructs were confirmed by dideoxynucleotide sequencing, and the expression of fusion proteins of the appropriate size was confirmed by Western blotting with anti-HA antibody (BAbCO). β-Galactosidase activity was visualized on 5-bromo-4-chloro-3-indoyl β-D-galactoside galactose-containing plates or measured in solution according to Current Protocols of Molecular Biology (31). Yeast cells were grown in the presence of glucose or galactose and resuspended in an equal volume of buffer Z (100 mM $NaPO_4$, 10 mM KC1, 1 mM $MgSO_4$, 50 mM β-mercaptoethanol). Cells were diluted 1:10 or 1:20 with buffer Z and permeabilized by SDS and chloroform. *o*-Nitrophenyl β-*p*-galactopyranoside was used as a substrate and the reaction was stopped when a medium-yellow color had developed. Activity was calculated according to: units = 1000 (A_{420}/A_{600} *tv*) when *t* = time and *v* = volume (17).

Affinity Precipitation and Peptide Competition Assays—NIH3T3 cells overexpressing the EGFR/Ret chimera or EGFR were treated with 100 nM EGF for 10 min at 37 °C. Treated and untreated cells were lysed with a solution containing 50 mM Hepes (pH 7.4), 1% Triton X-100, 10% glycerol, 150 mM NaCl, 5 mM KCl, 1 mM EDTA, 2 mM phenylmethylsulfonyl fluoride, 1 mM Na_3VO_4, 10 mM benzamidine, 10 μg/ml of aprotinin and leupeptin. Rat1 cells (HIRC) overexpressing InsR (18) and 293 cells expressing Enigma or Ret/ptc2 were lysed in a similar fashion. The 293 cells were transfected with the HA-Enigma or deletion mutants using the calcium phosphate precipitation procedure (32). Expression of Ret/ptc2 and mutant Ret/ptc2 was accomplished similarly. Cells were harvested 48 h after transfection. The lysates were incubated with GST fusion proteins that were immobilized on glutathione agarose beads for 1 h at 4 °C with continuous agitation. For assay of peptide competition, the indicated concentrations of peptides were mixed with lysates prior to incubation with GST fusion proteins. Beads were then washed four times with lysis buffer. Material bound to beads was resolved by electrophoresis and probed with antibodies to Ret, InsR, Enigma, phosphotyrosine, or HA.

RESULTS

Identification of the Recognition Motif for LIM3 of Enigma—To determine the recognition motif for the LIM3 domain of Enigma, a random peptide library selection technique was used to study the consensus binding site. Random peptide library selection has been successfully used to determine the sequence specificity of the peptide-binding sites of SH2 and SH3 domains as well as the optimal substrates of protein kinases (33, 34). We constructed a fixed tyrosine peptide library comprising peptides of the sequence: Met-Ala-*X*-*X*-*X*-*X*-Tyr-*X*-*X*-*X*-*X*-Ala-Lys-Lys-Lys, where *X* indicates all amino acid except Trp, Cys, Ser, Thr, or Tyr. Trp and Cys were omitted to avoid problems with sequencing and oxidation. The total theoretical degeneracy of this library is 15^8. The Met-Ala sequence at the amino terminus provides two amino acids to verify that peptides from this mixture are being sequenced. Sequencing of these two residues also provides quantitation of the peptides present. Ala-12 provides a second quantitation and an estimate of how much peptide loss occurred during sequencing. The poly-Lys tail prevented wash-out during sequencing and improved the solubility of the mixtures.

LIM3 of Enigma was expressed as a GST fusion protein in *Escherichia coli*. The fusion protein was immobilized on glutathione agarose and incubated with the tyrosine peptide library. Unbound peptides were washed away and bound peptides were released by acid and subjected to micro-sequencing. The amino acids preferentially selected by LIM3 of Enigma at positions −4, −3, −2, −1 amino-terminal to the Tyr residue and +1, +2, +3, +4 carboxyl-terminal to the Tyr residue are shown in Fig. 1*A*. The greatest selectivity was observed at the −1 and +2 positions where Pro was preferred. At the −2 position, glycine was the preferred amino acid and at the +3 position, both Val and Ile were highly selected. Phe was preferred at position +4.

Because Pro was preferred at positions −1 and +2, a second library with the sequence Met-Ala-*X*-*X*-*X*-*X*-*X*-Pro-*X*-*X*-Pro-*X*-*X*-*X*-Ala-Lys-Lys-Lys in which Pro was fixed with two intervening amino acids was designed to further test selectivity. This library lacks only Cys and Trp and has a degeneracy of 18^{10}.

A

Tyrosine Random peptide Library

-4	-3	-2	-1	0	+1	+2	+3	+4
E(1.7)	E(1.7)	G(1.5)	P(2.0)	Y	G(1.3)	P(1.9)	V(2.4)	F(1.4)
I(1.4)	I(1.4)				H(1.3)		I(2.3)	H(1.4)
V(1.4)								

B

Proline Random peptide Library

-4	-3	-2	-1	0	+1	+2	+3	+4	+5
X	X	G(2.1)	P	M(1.6)	G(1.4)	P	I(1.9)	Y(1.7)	A(1.7)
				F(1.6)			M(1.5)	F(1.4)	
				Y(1.6)			V(1.3)		
				I(1.4)					

C

Consensus:	G	P	Hyd	G	P	Hyd	Y/F	A
Exon16/InsR:	G	P	L	G	P	L	Y	A

FIG. 1. **Substrate specificity of LIM3 of Enigma determined by binding degenerate peptide libraries.** Values in *parentheses* indicate the relative selectivity for the amino acids and *X* indicates no selectivity. The one-letter amino-acid code is used. LIM3 of Enigma was expressed as a GST fusion protein and immobilized on glutathione agarose. A Tyr-fixed random peptide library (*A*) or a Pro-fixed random peptide library (*B*) was presented to the immobilized GST-LIM3/Enigma. The unbound peptides were washed away and the retained peptide mixture was sequenced. The consensus peptide sequence is compared to the endocytic code of exon 16 of InsR (*C*).

This library also included Tyr at the 10 degenerate positions. The general motif determined by this library was similar to those found with the Tyr-fixed peptide library (Fig. 1*B*). In addition, a Tyr residue was highly selected at position +4. By comparison of these two motifs from two peptide library selections, the peptide sequence of Gly-Pro-Hyd-Gly-Pro-Hyd-Tyr/Phe-Ala was determined to be the recognition motif for LIM3 of Enigma (Fig. 1*C*). This peptide sequence is highly homologous to the sequence of exon 16 of InsR.

To confirm the binding motif for LIM3/Enigma, peptides were tested for their capacity to disrupt the complex of LIM3/Enigma with holo InsR. HIRC cell lysates containing InsR were incubated with GST-LIM3/Enigma without or with competitor peptides. As shown in Fig. 2, binding of InsR to GST-LIM3/Enigma was inhibited by a 12-amino acid peptide (Asp-Gly-Pro-Leu-Gly-Pro-Leu-Tyr-Ala-Ser-Ser-Asn) corresponding to exon 16 of the InsR but not by mutant peptides (Asp-Gly-Pro-Leu-**Ala**-Pro-Leu-**Ala**-Ala-Ser-Ser-Asn and Asp-Gly-**Ala**-Leu-Gly-**Ala**-Leu-Tyr-Ala-Ser-Ser-Asn). The single substitution of the Leu immediately preceding the Tyr for Ile did not affect its ability to compete for InsR binding, confirming the random peptide library selection of a hydrophobic residue at position +3. These peptide competition results demonstrate that both the Pro and Tyr residues are required to mediate interaction of LIM3 of Enigma with exon 16 of InsR.

Differential Recognition of Ret/ptc2 and InsR by LIM Domains of Enigma—When an oncogenic form of Ret, Ret/ptc2, was used in a yeast two hybrid screen to identify interacting proteins, several SH2 domain containing proteins and Enigma were isolated (25). To determine the domains of Enigma responsible for this interaction with Ret/ptc2, regions of Enigma were cloned into pJG4–5 and tested for their ability to bind Ret/ptc2 expressed as a LexA fusion protein in pEG202 in yeast. As shown in Fig. 3*A*, full-length Enigma bound similarly to Ret/ptc2 and exon 16/InsR. The amino-terminal 279 amino acids of Enigma did not interact with either Ret/ptc2 or exon 16/InsR. The carboxyl-terminal 275 amino acids containing the three LIM domains were thus responsible for protein·protein interactions with both Ret and InsR. When the three LIM domains of Enigma were divided into individual LIM domains, LIM2 of Enigma bound Ret/ptc2 but not exon 16/InsR. Conversely, LIM3 of Enigma bound exon 16/InsR but not Ret/ptc2. These results demonstrate that LIM2 of Enigma was responsible for the association of Enigma with Ret/ptc2 and could be physically separated from LIM3 of Enigma which was responsible for Enigma association with InsR. LIM1 of Enigma, which bound two atoms of zinc characteristic of LIM domains[3] did not associate with either RET/ptc2 or InsR.

The specificity of interaction of Ret/ptc2 and InsR with other LIM domains was further examined. As shown in Fig. 3*B*, LIM domains of Mec-3, Isl-1, Lmx-1, zyxin, CRP, and paxillin did not recognize Ret/ptc2 or exon 16/InsR. The specificity for recognition thus resides in the LIM domains of Enigma.

Mapping the Interaction Site of of Ret with Enigma—Deletion of the carboxyl terminus of Ret/ptc2 distal to the conserved tyrosine kinase core abolished both mitogenic activity and Enigma binding (25). To determine whether the carboxyl-terminal region of Ret is sufficient to support the interaction, the carboxyl-terminal 61 amino acids of Ret/ptc2 (residues 536 to 596) were expressed as a GST fusion protein (GST-C′/Ret) and tested for their ability to bind Enigma. GST and GST-C′/Ret were immobilized on glutathione agarose and mixed with full-length, amino-terminal or carboxyl-terminal domains of Enigma that were expressed as HA epitope-tagged fusion proteins in 293 cells (Fig. 4*A*). Equal amounts of GST and GST-C′/Ret were assessed for their ability to bind these Enigma proteins (Fig. 4*B*). GST-C′/Ret bound full-length Enigma and

[3] D. Winge, personal communication.

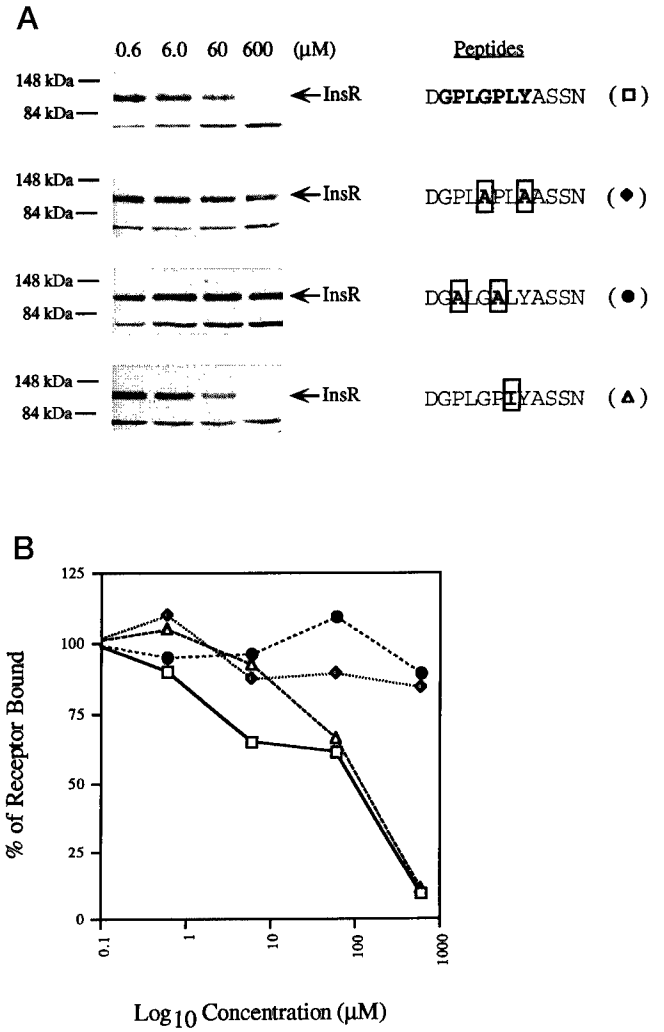


FIG. 2. **Peptide competition with InsR binding to LIM3 of Enigma (*A*) lysates of Rat1 cells that overexpressed InsR were incubated with GST-LIM3/Enigma in the presence of varying concentrations of the indicated peptides.** GST beads were washed and the associated InsR eluted and detected by Western blotting using an antibody specific to the β subunit of InsR. *B*, bound receptor was quantitated by scanning densitometry and plotted against the concentration of competitor peptide. The amount bound in the absence of competitor peptide was set at 100%.

the carboxyl terminus containing the three LIM domains of Enigma but failed to bind the amino terminus of Enigma. There was no binding of any of the three forms of Enigma protein to GST. These results indicate that the carboxyl-terminal 61 amino acids of Ret contain the motif that is required for the association of Ret with Enigma.

To map the binding site within the carboxyl terminus of Ret, peptides were used to specifically disrupt the complexes of Ret with Enigma. Four 20-amino acid peptides (Fig. 5*A*) were assayed for their ability to disrupt the interaction of Ret with Enigma. Full-length Enigma was mixed with the indicated peptides and interacted with immobilized GST-C′/Ret. As shown in Fig. 5B, incubation with wild type peptide, Asn-Lys-Leu-Tyr, and mutant peptide, Asn-Lys-Leu-**Phe**, were sufficient to displace Enigma binding to the carboxyl terminus of Ret, while the Ret mutant peptide **Ala**-Lys-Leu-**Ala** and the peptide corresponding to exon 16/InsR were without effect. These results indicate that the sequence **Asn-Lys-Leu-Tyr** in the carboxyl terminus of Ret is necessary for interaction with the LIM2 domain of Enigma. Mutation of the Asn and Tyr residues abolished interaction but a Phe substitution for Tyr was tolerated.

Specificity was further assessed by using these peptides to disrupt interaction of an EGFR/Ret chimera (26) with immobilized GST-LIM2 of Enigma. NIH3T3 cell lysates expressing EGFR/Ret were incubated with GST-LIM2/Enigma in the presence of the indicated peptides, and bound receptors were detected by an anti-Ret antibody. Similar to the results in Fig. 5*B*, peptides Asn-Lys-Leu-Tyr and Asn-Lys-Leu-**Phe** displaced EGFR/Ret binding to LIM2/Enigma (Fig. 5*C*). However, the peptide with the two amino acid mutation to **Ala**-Lys-Leu-**Ala** and the exon 16/InsR peptide failed to compete for the binding, confirming the Asn-Lys-Leu-Tyr sequence at the carboxyl terminus of Ret is the core recognition site for LIM2/Enigma. EGFR alone did not interact with Enigma (data not shown).

Although the mutant peptide Asn-Lys-Leu-**Phe** blocked the interaction of Enigma and Ret using GST-fusion protein assays, mutation of Tyr^{586} to Phe in Ret/ptc2 decreased this interaction as assayed in a yeast two hybrid system and decreased Ret/ptc2-stimulated DNA synthesis in microinjection experiments in mouse fibroblasts (25). To clarify these differing results, the effects of replacement of Tyr^{586} with Phe in Ret/ptc2 on Enigma binding were quantitated. Wild-type Ret/ptc2 (Tyr^{586}) and mutant Y586F Ret/ptc2 (Phe^{586}) were expressed in 293 cells and the relative affinities of these proteins for the LIM domains of Enigma were measured. As shown in Fig. 5*D* the affinity of Tyr^{586} exceeded that of Y586F Ret/ptc2 for Enigma by approximately 5-fold. Deletion of the carboxyl terminus containing this region, *i.e.* Ret/ptc2 truncated at residue 574 completely abolished the interaction (data not shown). LIM2 of Enigma thus recognized the Phe substituted carboxyl terminus of Ret but with lower affinity compared to wild type Ret/ptc2 with the Tyr-containing sequence. The differing results using the yeast two hybrid system and *in vitro* peptide competition are explained by the decreased affinity of Y586F compared to wild type Ret/ptc2 for LIM 2 of Enigma.

Tyrosine Phosphorylation of Ret Is Not Required for Binding to LIM2 of Enigma—To investigate whether tyrosine kinase activation was required for the association between LIM2/Enigma and Ret/ptc2, the EGFR/Ret chimeric protein was used. Because the ligand for the Ret tyrosine kinase receptor is unknown, the chimera generated by fusing the extracellular and transmembrane domains of EGFR and the intracellular domain of Ret was used (Fig. 6*A*). EGF activated the Ret tyrosine kinase activity and mitogenic responses of this chimera (26). NIH3T3 cells overexpressing EGFR/Ret were treated without or with EGF and cell lysates were mixed with GST or GST-LIM domains of Enigma. As shown in Fig. 6*B*, only GST-LIM2/Enigma interacted with EGFR/Ret. GST, GST-LIM1, or GST-LIM3 of Enigma did not bind. Ligand treatment did not effect the binding of EGFR/Ret to LIM2 of Enigma (*left panel*). Phosphorylation of the EGFR/Ret chimera was also examined by Western blotting using an anti-phosphotyrosine antibody. GST-LIM2 of Enigma interacted with phosphorylated as well as unphosphorylated EGFR/Ret receptors. The interaction of LIM2 of Enigma with Ret is thus independent of ligand activation and Ret autophosphorylation.

DISCUSSION

The growing number of proteins that contain one or more LIM domains function in a variety of pathways and locations within the cell, implicating LIM domains as versatile protein modules that are capable of acting in diverse cellular contexts. Although the NMR structure of LIM2 of CRP resembles the DNA binding domain of the GATA-1 transcription factor, no direct evidence that a LIM domain binds to nucleic acids has been presented. Indeed, a lack of affinity for target DNA sequences has been reported for the LIM domains of Mec-3 (35).

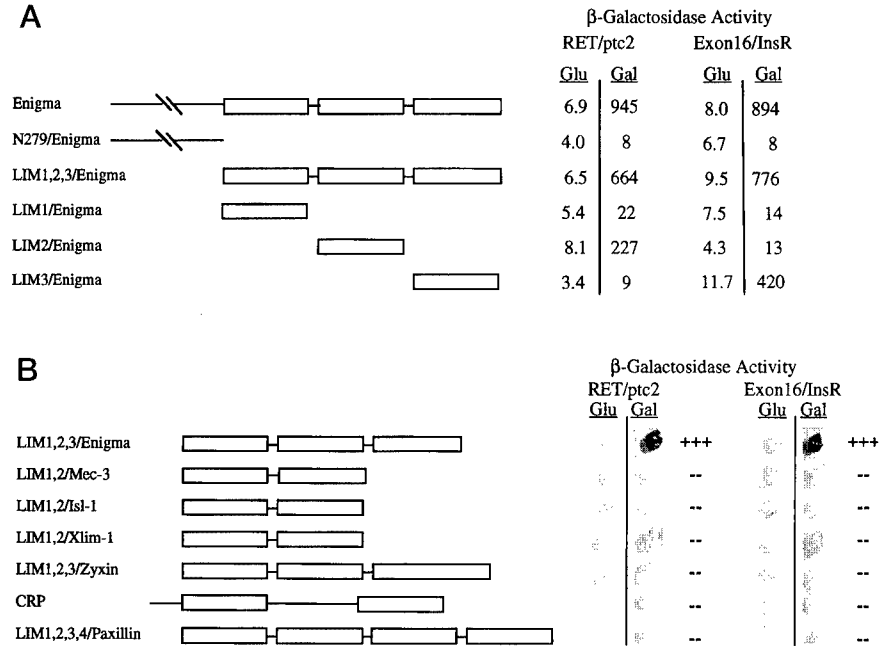


FIG. 3. **Specific interaction of LIM domains of Enigma with Ret/ptc2 and exon 16/InsR in a yeast two hybrid system.** *A*, differential recognition of Ret/ptc2 and exon 16/InsR by LIM2 and LIM3 of Enigma. *B*, comparison of Ret/ptc2 and exon 16/InsR interaction with LIM domains of Enigma and other proteins. Ret/ptc2 and exon 16 InsR were expressed as LexA DNA binding domain fusion proteins in plasmid pEG202. β-Galactosidase assays were performed on yeast expressing individual LexA fusion proteins and the indicated B42 activation domain fusion proteins in plasmid pJG4-5. β-Galactosidase activity of each transformant was visualized and measured in solution. "+" indicates dark blue and "−" indicates white colonies. Numbers indicate β-galactosidase activity units quantitated from solution assays.

Most available evidence indicates that LIM domains function in specific protein-protein interactions (1, 8).

The present studies demonstrate that LIM2 of Enigma specifically interacts with Ret while LIM3 of Enigma specifically interacts with InsR. The Asn-Lys-Leu-Tyr sequence at the carboxyl terminus of Ret was essential for the formation of the Ret-Engima complex. For the interaction of InsR with Enigma, the Gly-Pro-Leu-Gly-Pro-Leu-Tyr sequence of the juxtamembrane region of InsR was required. Both LIM2 and LIM3 recognized Tyr-containing motifs located outside of the tyrosine-kinase cores of Ret and InsR. Although the recognition motifs for LIM2 and LIM3 of Enigma share sequence similarity, they were not exchangeable, demonstrating that the two LIM domains have the ability to distinguish between two Tyr-based motifs. These results also indicate that individual LIM domains within a single protein have distinct partner preferences. Because the structural features of LIM domains are highly conserved, sequences other than the conserved residues that are involved in metal coordination must be important for defining the selectivity of individual LIM domains for their particular partner. LIM1 of Enigma failed to recognize either Ret or InsR and is likely to have a yet unidentified target protein in cells.

Tyr-based motifs serve a number of functions. Tyr-containing tight turns are the essential structural feature of the endocytic codes of many proteins (19). Four to six amino acid sequences containing an essential Tyr residue also function as lysosomal and trans-Golgi to basolateral surface targeting codes (36–38). There is no evidence for covalent modification of Tyr residues within these trafficking codes. Phosphorylated Tyr residues in specific sequence contexts serve as the recognition motif for SH2 and PTB domains (33, 39, 40). The present studies indicate that Tyr-containing sequences also function as recognition elements for certain LIM domains. The Gly-Pro-Leu-Tyr motif of InsR that is recognized by LIM3 of Enigma forms a Tyr-containing tight turn (20). Use of two random

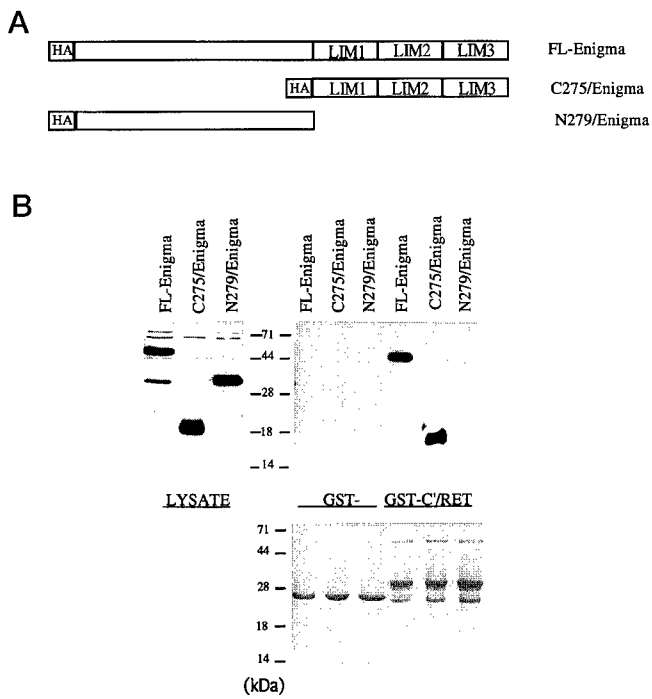
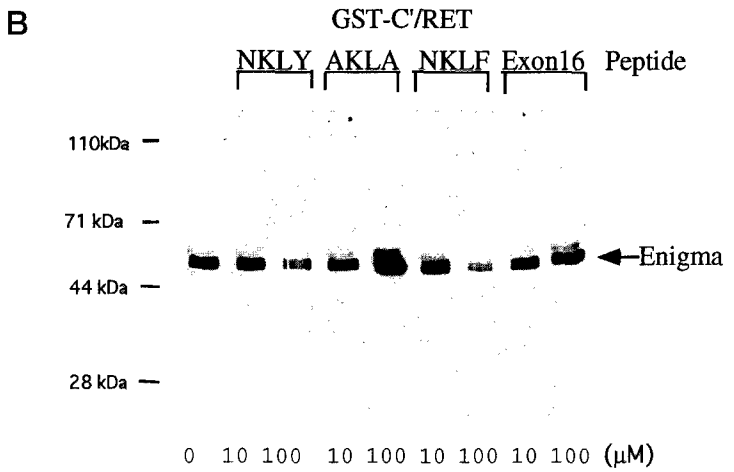
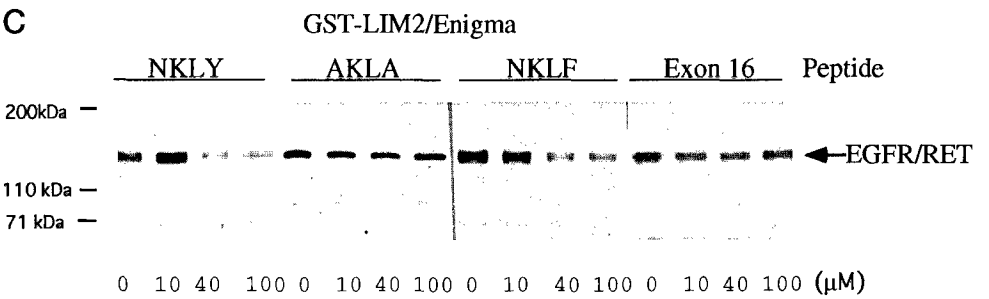


FIG. 4. **Direct association of Enigma with the carboxyl terminus of Ret.** *A*, schematic representation of the Enigma fragments used in the binding assays. Full-length and regions of Enigma were expressed as HA epitope-tagged fusion proteins. *B*, binding of Enigma to the carboxyl-terminal 61 amino acids (residues 536–596) of Ret. 293 cells were transfected with the indicated expression vectors; cell lysates were prepared 48 h later and incubated with GST or GST-C′/Ret. Bound material was analyzed by Western blotting with antibody to the HA epitope (*upper*). The *bottom panel* is a Coomassie Blue-stained gel to quantitate GST and GST fusion protein used in the assays. The *left panel* shows the amount of proteins in the lysate prior to interaction with GST C′/Ret.

A

Peptide	Amino Acid Sequence
NKLY	PSTWIE**NKLY**GRISHAFTRF
AKLA	PSTWIE**AKLA**GRISHAFTRF
NKLF	PSTWIE**NKLF**GRISHAFTRF
Exon16	DGPLGPLYASSNPEYLSASD





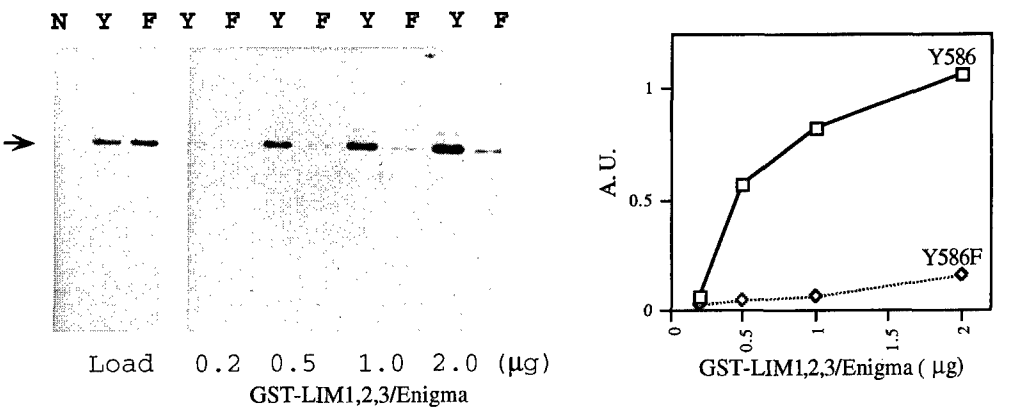


FIG. 5. **Sequence specificity of Ret binding to Enigma using peptide competition assays and GST-fusions.** *A*, amino acid sequences of the peptides used for competition. The NKLY peptide corresponds to the carboxyl-terminal 20 amino acids (residues 577–596) of Ret/ptc2. Exon 16 corresponds to the juxtamembrane region of InsR. *B*, specific dissociation of Enigma and GST-C′/Ret complexes by peptides. The HA-tagged Enigma protein was expressed in 293 cells and mixed with GST-C′/Ret without or with the indicated concentrations of peptides. Bound Enigma protein was detected by anti-HA antibody. *C*, peptide competition of EGFR/Ret binding to GST-LIM2 of Enigma. Lysates were prepared from NIH3T3 cells expressing the EGFR/Ret chimera and bound receptor was detected using the anti-Ret antibody. *D*, comparison of binding of Tyr^{586} with Y586F Ret/ptc2 to the carboxyl terminus of Enigma. Wild-type Ret/ptc2 (Tyr^{586}) and the point mutation Y586F Ret/ptc2 (Phe^{586}) were expressed in 293 cells. Equal amounts were mixed with the indicated amount of GST-LIM1,2,3/Enigma, and bound Ret/ptc2 was detected by the anti-Ret antibody (*left*). Bound protein was quantitated by scanning densitometry and plotted against the amount of GST LIM domains of Enigma used (*right*). The amount bound is expressed as absorbance units (*A.U.*)

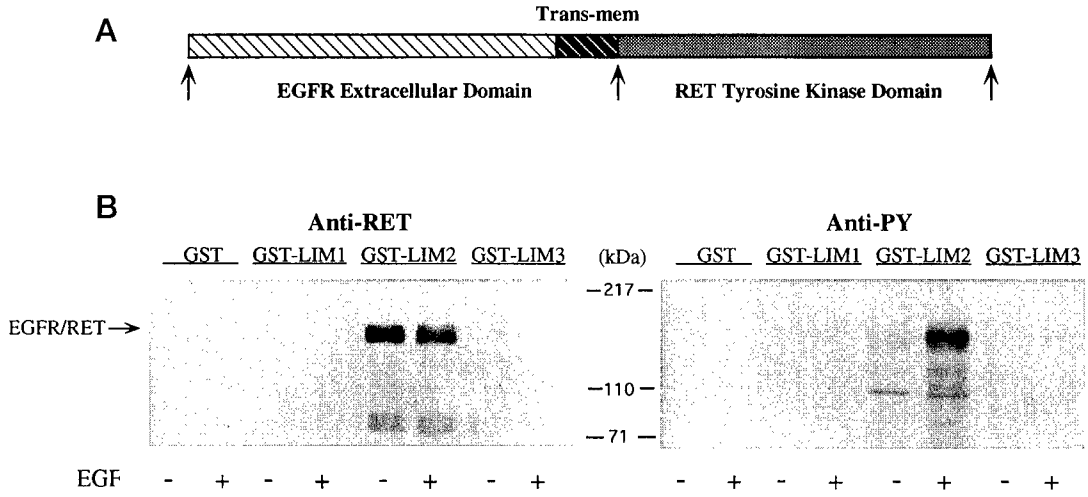


FIG. 6. **Specificity of binding of the LIM2 domain of Enigma to EGFR/Ret *in vitro*.** *A*, schematic structure of the EGFR/Ret chimera indicating the extracellular and transmembrane domains of EGFR and the intracellular domain of Ret. The chimeric receptor utilized EGF as a ligand to activate its tyrosine kinase. *B*, immunoblot analysis of the binding of EGFR/Ret chimera by GST fusion proteins. NIH3T3 cells overexpressing the EGFR/Ret chimera were treated without or with 100 nM EGF. Cell lysates were incubated with GST or GST-LIM domains of Enigma. Bound proteins were analyzed by Western blotting with antibodies to Ret or to phosphotyrosine.

peptide libraries indicated that the target recognized by LIM3 of Enigma consisted of the more extended sequence Gly-Pro-Leu-Gly-Pro-Leu-Tyr-Ala. The Asn-Lys-Leu-Tyr motif of Ret that is recognized by LIM2 of Enigma resembles the endocytic sequence in the LDL receptor which forms a Tyr-containing tight turn (41).

The function of the target sequences in Ret and InsR are different. LIM3 of Enigma recognized the major endocytic code of InsR while LIM2 of Enigma interacted with the carboxyl terminus of Ret. Ret is a protein tyrosine kinase receptor implicated in several disease processes. Mutations that inactivate its tyrosine kinase result in Hirschsprung's disease characterized by defective sympathetic innervation of the large intestine (42, 43). Activating mutations characterize a group of inherited multiple endocrine neoplasia type syndromes that include MEN2A, MEN2B, and familial medullary thyroid cancer (44, 45). Gene rearrangements including the one fusing the type 1 regulatory subunit of cyclic AMP-dependent protein kinase to the tyrosine kinase domain of Ret (Ret/ptc2) occur as oncogenic events in papillary thyroid carcinoma (28). In a nuclear microinjection assay the mitogenic activity of Ret/ptc2 was abolished by carboxyl-terminal truncation to residue 574 or by the mutation Y586F (25, 29). Deletion of sequences distal to residue 574 abolished interaction with Enigma and mutation of Tyr^{586} reduced the affinity of Enigma for Ret. Additionally, co-expression of the LIM domains of Enigma blocked the mitogenic activity of Ret/ptc2, implicating involvement of Enigma in the mitogenic signaling of Ret (25). The ability of LIM3 of Enigma to recognize the active endocytic codes of InsR fulfills the first property of the endocytic mechanism, but additional functional criteria will be necessary to critically test the hypothesis that Enigma functions in endocytosis of InsR.

Interactions of LIM domains of Enigma with these two receptors did not require either tyrosine kinase activity or tyrosine autophosphorylation on their target sequences. However, for both endocytosis of InsR and mitogenic signaling by Ret, activation of tyrosine kinase activity is necessary. Interactions of Enigma with InsR and Ret are thus proposed to be necessary but not sufficient to support these biological processes. Interestingly, Tyr^{1062} in holo Ret corresponding to Tyr^{586} in Ret/ptc2 is phosphorylated when expressed in COS cells (46). The stoichiometry was not determined but this could provide a mechanism for the reversible association of Ret with Enigma.

Most LIM proteins contain more than one LIM domain (1, 8). Finding distinct targets for two LIM domains of a single protein not only supports the hypothesis that LIM domains function in protein·protein interactions but indicates a possible adaptor function to assemble multiple proteins into a complex. Alternatively, multiple LIM domains could function to differentially assemble proteins with distinct receptors. The finding that binding of InsR and of Ret to Enigma does not require the protein tyrosine kinase activity of either receptor distinguishes LIM domain interactions from those of SH2 and PTB domains which direct assembly that is dependent on tyrosine kinase activity and covalent modifications of proteins (33, 39). Because the processes requiring the target sequences in InsR and Ret that are recognized by Engima do depend on the tyrosine kinase activity of these receptors, mechanisms of function of the assembled LIM domain complexes must coordinate with tyrosine kinase activity perhaps via substrate phosphorylation or protein assemblies.

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Communication

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Tyrosines outside the Kinase Core and Dimerization Are Required for the Mitogenic Activity of RET/ptc2*

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Defects in the c-*ret* proto-oncogene, a member of the protein tyrosine kinase receptor family, have recently been linked to two types of genetic syndromes, Hirschsprung's disease and the multiple endocrine neoplasia family of inherited cancers. RET/ptc2 is the product of a papillary thyroid carcinoma translocation event between the genes coding for c-*ret* and the type Iα regulatory subunit of protein kinase A (RIα) (Lanzi, C., Borrello, M., Bongarzone, I., Migliazza, A., Fusco, A., Grieco, M., Santoro, M., Gambetta, R., Zunino, F., Della Porta, G., and Pierotti, M. (1992) *Oncogene* 7, 2189–2194). The resulting 596-residue protein contains the first two-thirds of RIα and the entire tyrosine kinase domain of c-*ret* (RET_{tk}). An *in vivo* assay of growth stimulatory effects was developed, which consisted of microinjecting a RET/ptc2 expression plasmid into the nuclei of 10T1/2 mouse fibroblasts and observing the incorporation of 5-bromodeoxyuridine. This assay was used to determine that only the dimerization domain of RIα fused to RET_{tk} is required for RET/ptc2's mitogenic activity. In addition, all of the reported Hirschsprung's disease point mutations in the RET_{tk} (S289P, R421Q, and R496G) inactivate RET/ptc2 in our assay, confirming that these are loss of function mutations. Two tyrosines outside the conserved kinase core were also identified that are essential for full mitogenic activity of RET/ptc2. These two tyrosines, Tyr-350 and Tyr-586, are potential sites for Src homology 2 and phosphotyrosine binding domain interactions.

The *ret* oncogene was discovered in transfection experiments where DNA from human lymphomas was introduced into NIH3T3 cells (1). This oncogene was not expressed in the original lymphoma, but instead had arisen from a rearrangement during the transfection, hence the name *ret*. The c-*ret* proto-oncogene encodes a novel receptor tyrosine kinase with a cadherin-like motif in its extracellular domain (2).

The c-*ret* proto-oncogene is responsible for four human disease syndromes: Hirschsprung's disease, a developmental disorder, and the dominantly inherited cancer syndromes FMTC,[1] MEN2A, and MEN2B. Hirschsprung's disease occurs at a rate of approximately 1 in 6000 live births and is characterized by a lack of parasympathetic innervation of the lower intestine. Occurrence of this disease corresponds to c-*ret* gene deletions and nonsense point mutations leading to truncation of the expressed protein (3, 4). Also, three mutations resulting in single amino acid substitutions in the kinase region of the c-*ret* gene have been reported in Hirschsprung's patients (3, 4). In addition to medullary thyroid carcenoma, the unique pathologic feature of FMTC, MEN2A and MEN2B patients display additional hyperplasias. These syndromes have been linked to point mutations in the c-*ret* gene (5, 6, 7). Recent evidence suggests that, in the case of the MEN2A, these mutations result in the expression of constitutively activated forms of c-*ret* (8).

It has also been demonstrated that constitutively active forms of the c-*ret* proto-oncogene are present in nearly half of papillary type thyroid carcinomas (9). These oncogenic forms of *ret* are the result of somatic chromosome translocations or inversions, which fused the c-*ret* tyrosine kinase domain (RET_{tk}) with different genes. One of the resultant transforming sequences, RET/ptc2 (papillary thyroid carcinoma), was the product of a crossover between the genes coding for c-*ret* and the type Iα regulatory subunit of cyclic-AMP-dependent protein kinase (RIα) (10). The chimeric gene encodes a protein of 596 residues, which contains the first two-thirds of RIα and the entire tyrosine kinase domain of c-*ret* (11). RET/ptc2 is transforming, presumably due to constitutive tyrosine kinase activity, but the structural basis for RET_{tk} activation via fusion to RIα remains unclear.

Here we report the development and use of a microinjection assay to study the mitogenic activity of RET/ptc2. Deletion mutants were tested in the assay to determine which portions of RIα were required to elicit the mitogenic activity of RET/ptc2. Only the amino-terminal region of RIα, which encodes a dimerization domain (12), fused to the RET_{tk} was necessary. This portion of RIα was also capable of activating the tyrosine kinase domain of the epidermal growth factor receptor in this assay system. In addition, all of the reported Hirschsprung's disease point mutations in the RET_{tk} inactivated RET/ptc2 in our assay. To begin the search for interaction sites with other signaling proteins, the mitogenic activity of RET/ptc2 mutants lacking single tyrosines was tested. Two tyrosines were found to be essential for the mitogenic activity of RET/ptc2.

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[1] The abbreviations used are: FMTC, familial medullary thyroid carcinoma; MEN, multiple endocrine neoplasia; RIα, type Iα regulatory subunit of cAMP-dependent protein kinase (protein kinase A); RET_{tk}, tyrosine kinase domain of the c-Ret receptor; SH2, Src homology 2 domain; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; BrdUrd, bromodeoxyuridine; EGF, epidermal growth factor.

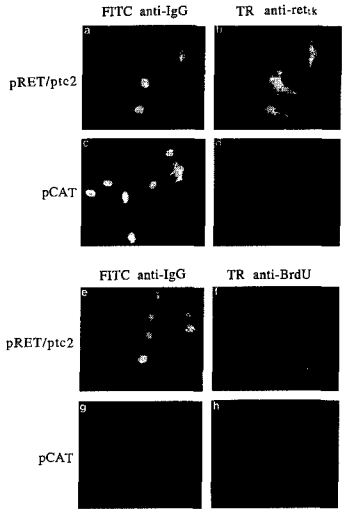

FIG. 1. **Immunostaining for RET/ptc2 expression and 5-bromodeoxyuridine (BrdUrd) incorporation in 10T1/2 cells injected with pRET/ptc2 (*a* and *b*, *e* and *f*) or pCAT (*c* and *d*, *g* and *h*) expression vectors.** *a*, IgG injection marker stained with FITC anti-guinea pig IgG. *b*, same field of cells as *a*, but showing Texas Red anti-RET$_{tk}$ staining. *c*, IgG injection marker stained with FITC anti-guinea pig IgG. *d*, Same field of cells as *c*, but showing Texas Red anti-RET$_{tk}$ staining. *e*, IgG injection marker stained with FITC anti-rabbit IgG. *f*, Same field of cells as *e*, but showing Texas Red anti-BrdUrd staining. *g*, IgG injection marker stained with FITC anti-rabbit IgG. *h*, Same field of cells as *g*, but showing Texas Red anti-BrdUrd staining.

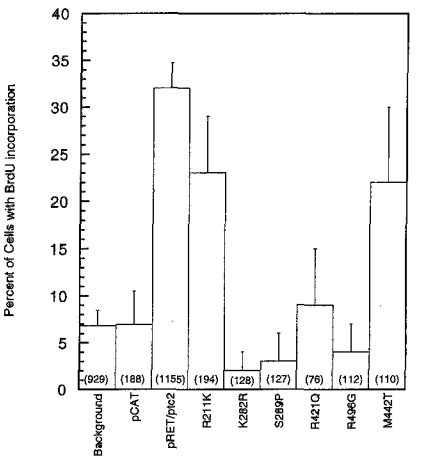

FIG. 2. **Mitogenic activity of RET/ptc2 point mutants.** The fraction of injected cells that stained positive for BrdUrd incorporation is shown for various plasmids expressing either wild-type RET/ptc2 or RET/ptc2 point mutants. In each case the plasmids were injected at a concentration of 100 μg/ml. The column labeled background represents uninjected cells under the assay conditions. The error bars show the 95% confidence interval calculated using the standard error of proportion. The *number* in *parentheses* is the total number of injected cells.

EXPERIMENTAL PROCEDURES

Construction of Mammalian Expression Plasmids—The cDNA coding for wild-type RET/ptc2 was excised from a pMAM-neo expression vector previously described (11) using the restriction enzyme *Xba*I. This fragment was then subcloned into the *Xba*I restriction site of the pRc/CMV mammalian expression vector (Invitrogen). Restriction digests were performed to screen for orientation, and then the entire cDNA sequence was verified using dideoxy sequencing (13) with Sequenase version 2.0 (U. S. Biochemical Corp.).

Site-directed mutagenesis was performed with the Kunkle method (14) using the Mutagene kit (Bio-Rad). Constructs expressing deletion mutants of RET/ptc2 were made by introducing *Nhe*I restriction sites flanking the segment of DNA to be deleted, digesting with *Nhe*I, and then ligating the new ends back together. All mutant constructs were sequenced to verify mutagenesis. Supercoiled plasmid DNA expressing various constructs were prepared by double banding in cesium chloride gradients (15).

Cell Culture and Microinjection—Mouse 10T1/2 fibroblasts were plated in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (DMEM + 10% FBS). The cells were maintained at 37 °C in a 10% CO_2 atmosphere and split just before reaching confluence.

For microinjection, cells were plated on glass coverslips and grown to 70% confluence in DMEM + 10% FBS. The coverslips were then transferred to DMEM containing 0.05% calf serum. After 24 h of incubation in the FBS-free medium, 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 μg/ml expression plasmid DNA and 6 mg/ml either guinea pig or rabbit IgG (Sigma). All microinjection experiments were performed using an automatic micromanipulator (Eppendorf, Fremont, CA), with glass needles pulled on a vertical pipette puller (Kopf, Tujunga, CA).

Immunostaining—For detection of RET/ptc2 protein, cells were fixed in 3.7% formaldehyde 5 h after injection for 5 min, and then washed with phosphate-buffered saline. The cells were then incubated successively with rabbit anti-RET$_{tk}$ (dilution 1:500), biotinylated donkey anti-rabbit IgG (dilution 1:400, Jackson ImmunoResearch, West Grove, PA), Texas Red streptavidin (dilution 1:100, Amersham Corp.), and FITC anti-guinea pig IgG (dilution 1:100, Jackson). The anti-RET$_{tk}$ antibody is a rabbit polyclonal antibody raised against a synthetic peptide corresponding to residues 535–551 of RET/ptc2 conjugated to keyhole limpet hemocyanin (Calbiochem).

DNA synthesis was assessed through incorporation of the thymidine analog 5-bromodeoxyuridine (BrdUrd) and its subsequent detection by immunostaining (16). Following nuclear microinjection, 0.1% BrdUrd labeling reagent (Amersham) was added to the starvation medium (DMEM + 0.05% calf serum), and the cells were incubated for an additional 24 h. Cells were fixed in 95% ethanol, 5% acetic acid for 30 min, and then washed with phosphate-buffered saline. Incorporation of 5-bromodeoxyuridine was visualized by successively incubating the fixed cells with mouse anti-bromodeoxyuridine (undiluted, Amersham), biotinylated donkey anti-mouse IgG (dilution 1:500, Jackson), Texas Red streptavidin (dilution 1:100, Amersham), and FITC anti-rabbit IgG (dilution 1:100, Jackson).

RESULTS AND DISCUSSION

Development of Mitogenic Assay—To evaluate which structural features of RET/ptc2 are essential for its mitogenic activity, an expression plasmid microinjection assay was developed. This assay allows for both rapid screening of protein expression and evaluation of mitogenic activity. The expression vector, pRET/ptc2, was co-injected into nuclei of serum-starved 10T1/2 fibroblasts with rabbit or guinea pig IgG, which served as an injection marker. After incubation for 24 h in 5-bromodeoxyuridine (BrdUrd)-containing starvation medium, the cells were fixed and stained for the IgG injection marker and either for RET$_{tk}$ protein expression (Fig. 1, *b* and *d*) or DNA synthesis as assessed by BrdUrd incorporation (Fig. 1, *f* and *h*). An identical construct containing the chloramphenicol acetyltransferase gene (pCAT) was used as a negative control.

Cells injected with pRET/ptc2 expressed protein detectable by anti-RET$_{tk}$ antibodies as early as 5 h post-injection (Fig. 1*b*). The expressed protein was cytoplasmic, and the amount and distribution of expressed protein was indistinguishable from that observed in pRET/ptc2-injected cells for all of the mutants discussed in this paper (data not shown). A plasmid concentration of 100 μg/ml was chosen for injections. Under these conditions, over 30% of the pRET/ptc2-injected cells entered S phase, compared to less than 6% of the pCAT-injected cells (Fig. 2).

Consequences of Mutations in ret—Using this assay, the mitogenic activity of various RET/ptc2 point mutants was tested (Fig. 2). To determine whether cAMP binding was important for RET/ptc2 activity, Arg-211 was mutated to Lys. This change eliminates high affinity cAMP binding in the first binding site of RIα (17); however, this point mutation had no significant effect on RET/ptc2 mitogenic activity. In contrast, elimination of a conserved Lys in the kinase domain (18), K282R, elimi-

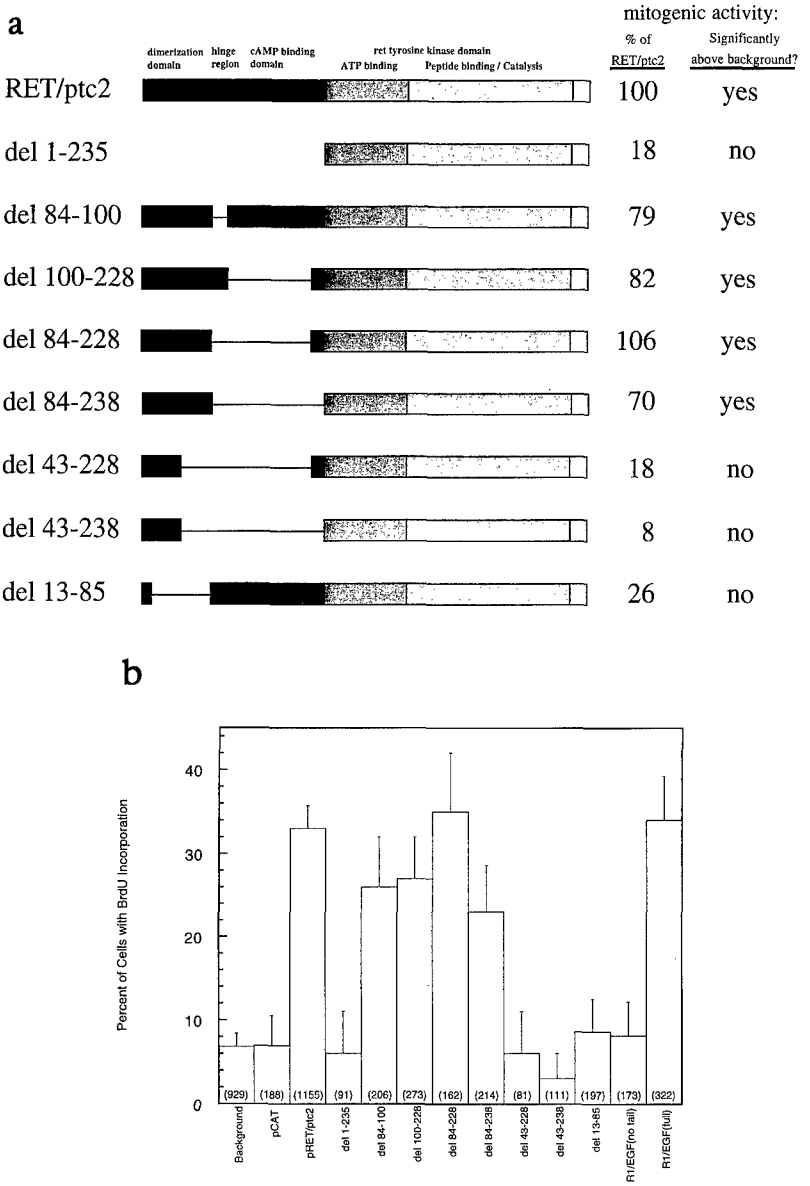

FIG. 3. **Mitogenic activity of RET/ptc2 deletion mutants.** *a*, schematic representation of the deletion mutants tested. In the RIα subunit the dimerization domain includes residues 1–84, the hinge region consists of residues 86–99, and the cAMP binding domain extends from residues 100 through 236. *b*, the fraction of injected cells that stained positive for BrdUrd incorporation is shown for various plasmids expressing either wild-type RET/ptc2, RET/ptc2 deletion mutants, RIα/EGF receptor kinase chimera, or RIα/EGF receptor kinase without its carboxyl-terminal tail (see text). Plasmids were injected at a concentration of 100 μg/ml. The error bars show the 95% confidence interval calculated using the standard error of proportion. The number in parentheses is the total number of injected cells.

nated *in vivo* mitogenic activity. Likewise, the three reported Hirschsprung's disease point mutations located in the c-Ret tyrosine kinase domain, S289P, R421Q, and R496G, all inactivated RET/ptc2 in this assay. These results support the model that Hirschsprung's disease results from a loss of c-Ret function and are consistent with recently obtained results using transfection assays on NIH3T3 and PC12 cell lines (19). The MEN2B mutation (M442T) had no effect on this activated form of c-Ret, which does not contradict a recent report that this mutation alters substrate specificity rather than catalytic activity of c-*ret* (8).

Role of RIα in RET/ptc2—The NH_2 terminus of RET/ptc2 comprises the first 236 residues of RIα, which includes the RIα dimerization domain, the cAMP-dependent protein kinase autoinhibitory site, and most of RIα's first cAMP binding domain (20). This is fused to the cytoplasmic portion of the RET receptor, which consists of its tyrosine kinase domain (RET_{tk}) followed by a short COOH-terminal tail. The wild-type RIα subunit is a disulfide-bonded dimer with the protomers aligned through residue 37 in an antiparallel orientation (12). Constructs expressing deletion mutants were tested to determine the role of RIα in the activation of RET_{tk} (Fig. 3). These mutants showed clearly that the only portion of RIα required for RET/ptc2 mitogenic activity was the dimerization domain (Fig. 3*a*) and support the hypothesis that the RET_{tk} is activated in RET/ptc2 via the dimerization domain of RIα. One model for receptor tyrosine kinase activation is through ligand-induced dimerization, or oligomerization, followed by trans-phosphorylation (21, 22). This would occur through a parallel orientation of dimerized receptors, since both monomers are membrane-bound. In contrast, the dimerization domain of RIα may provide an antiparallel orientation (12) for the linked monomers, and we are currently investigating the structural basis for this novel receptor tyrosine kinase activation mechanism *in vitro*.

To investigate whether RIα-mediated dimerization is a general activating motif for receptor tyrosine kinases, an analogous construct to RET/ptc2 was made by substituting the epidermal growth factor receptor tyrosine kinase (EGF_{tk}) for the RET_{tk}. The expression construct encoding RIα residues 1–236 fused to the EGF_{tk} and its COOH-terminal tail, residues 647-1186, was as active as pRET/ptc2 in our microinjection assay (Fig. 3*b*). The carboxyl-terminal tail, residues 959-1186, contains all of the known Src homology 2 domain (SH2) docking sites of the EGF receptor (23). Deletion of this tail in the RIα/EGF_{tk} construct eliminated its mitogenic response in our assay (Fig. 3*b*). COOH-terminal truncations of the holo-EGF

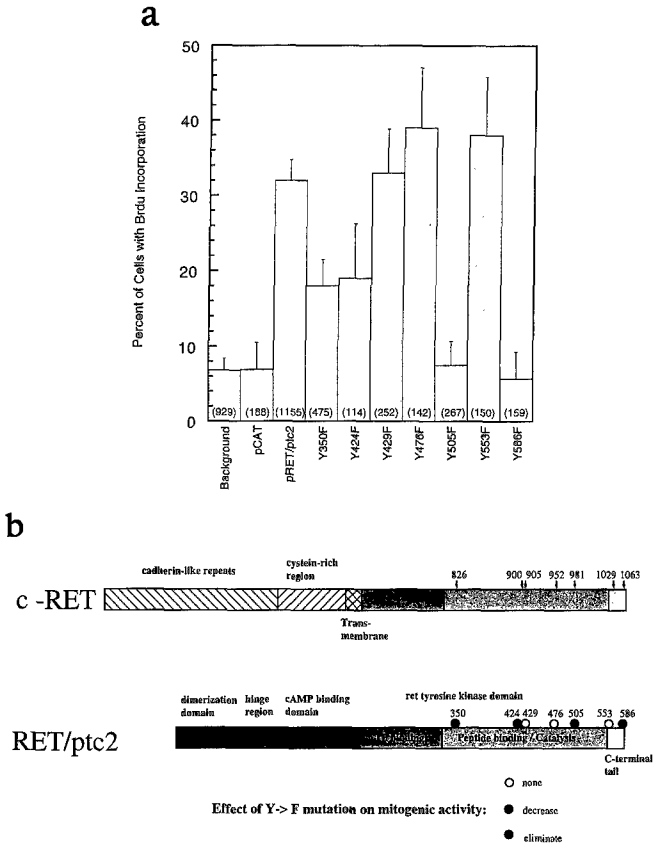

FIG. 4. **Mitogenic activity of RET/ptc2 tyrosine to phenylalanine mutants.** *a*, the fraction of injected cells which stained positive for BrdUrd incorporation is shown for various plasmids expressing either wild-type RET/ptc2 or RET/ptc2 Tyr to Phe point mutants. Plasmids were injected at a concentration of 100 μg/ml. The *column* labeled *background* represents uninjected cells under the assay conditions. The *error bars* show the 95% confidence interval calculated using the standard error of proportion. The *number* in *parentheses* is the total number of injected cells. *b*, schematic representation of RET/ptc2 showing the locations of the seven tyrosines mutated to phenylalanine. The kinase domain of RET extends from residues 237 to 545 of RET/ptc2, the COOH-terminal tail ends at residue 596, and the platelet-derived growth factor-like insert of RET encompasses residues 344–367. The corresponding tyrosines are shown on the c-Ret schematic (2).

receptor have been shown to have increased transforming activity, attributed to an inability to internalize and attenuate the EGF signal (24, 25). The complete loss of mitogenic activity for our COOH-terminal EGF_{tk} deletion suggests that SH2 domain interactions are more important for the mitogenic response of $RI\alpha/EGF_{tk}$ than for COOH-terminal truncations of the holo-EGF receptor.

Role of Tyrosines in RET/ptc2—To identify possible SH2 interaction sites in RET/ptc2, several tyrosines were mutated to Phe and the resultant mutants tested for mitogenic activity (Fig. 4). Of the seven Tyr mutants, two, Y350F and Y424F, exhibited significantly reduced mitogenic activity. Mutations at two other tyrosines, Y505F and Y586F, abolished the mitogenic effect. Tyr-350 is located in a variable insert region of receptor tyrosine kinases, and in the platelet-derived growth factor receptor this insert region contains two SH2 binding sites (26). Tyr-424 in RET/ptc2 corresponds to Tyr-1158 in the insulin receptor (26), and phosphorylation at Tyr-1158 is required for full activity (27). Tyr-505 is in a sequence that aligns with the H-helix of the insulin receptor tyrosine kinase (18, 28). This tyrosine is highly conserved in the receptor tyrosine kinases (26) and could serve a structural role rather than as an SH2 docking site. Tyr-586, which completely eliminated mitogenic activity when mutated to Phe, is in the carboxyl-terminal tail of the RET_{tk}.

Tyrosines 586 and 350 are the best candidates for residues that provide SH2 or phosphotyrosine binding domain docking sites essential for the mitogenic response of RET/ptc2. Mutation of the other Tyr in the COOH-terminal tail, Tyr-553, had no effect on mitogenic activity. Results from the Y586F mutant indicate that kinase activity alone is not sufficient for the mitogenic response of RET/ptc2. Work is under way to investigate RET/ptc2 phosphorylation sites and to search for interacting proteins.

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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 (RI) with the tyrosine kinase domain of c-Ret. Mitogenic activity of Ret/ptc2 required dimerization via the N terminus of RI 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).

Chromosome translocations or inversions in papillary thyroid carcinoma (ptc)[1] 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 (RI) gene with *c-ret* (9). This protein, Ret/ptc2, is 596 residues in length and contains the N-terminal two-thirds of RI 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 RI 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 EGFR/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 that interacts with Tyr-586 of Ret/ptc2. The SH2 domains of PLCγ and Grb10 were both found to interact with Ret, but neither bound at Tyr-586. Mutations in Ret/ptc2, which interfered with these SH2 interactions, had no effect on the mitogenic activity. A protein that interacted with Tyr-586 was identified as Enigma (13). This interaction was found to be highly specific, mediated by the second of the three LIM domains of Enigma and independent of Ret phosphorylation or activity. Disruption of the interaction of Ret/ptc2 with Enigma, either by mutation of Tyr-586 in Ret/ptc2 or by co-expression with a dominant negative form of Enigma, abolished the mitogenic activity of Ret/ptc2.

EXPERIMENTAL PROCEDURES

Two-hybrid Screen—A yeast two-hybrid screen was performed by the methods of Vojtek *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

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[1] The abbreviations used are: ptc, papillary thyroid carcinoma; RI, type Iα regulatory subunit of cyclic AMP-dependent protein kinase; PLCγ, phospholipase C-γ; GST, glutathione *S*-transferase; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; SH2, Src homology 2.

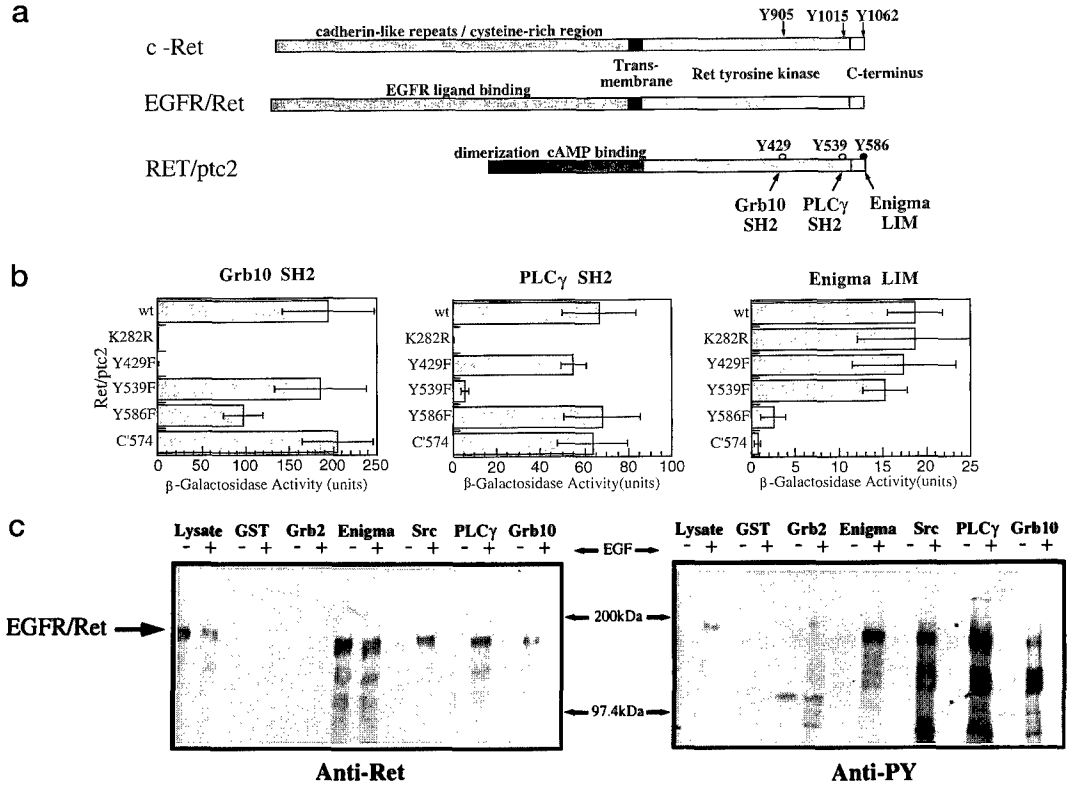

FIG. 1. **Requirements for interaction of Grb10, PLCγ, and Enigma with Ret.** *a*, schematic representation showing the location of tyrosines that are required for interaction with the SH2 domains of Grb10 and PLCγ and the second LIM domain of Enigma. The corresponding residues in c-Ret are indicated, and the EGFR/Ret chimera used in *panel c* is shown. *b*, analysis of mutant Ret/ptc2 interactions using the yeast two-hybrid system. The indicated mutant of Ret/ptc2 and Grb10 SH2, PLCγ SH2, or LIM 2/3 of Enigma were co-expressed in yeast, and interactions were measured using solution assays of the reporter β-galactosidase. Y586F also contained R588T. The mean ± S.D. (n = 6) for each interacting pair is shown. *c*, *in vitro* interactions between GST-fusion proteins and the EGFR/Ret chimeric receptor. Clonal NIH3T3 cells expressing an EGFR/Ret chimeric receptor were treated (+) or not treated (−) with EGF before lysis. Western blots of EGFR/Ret that bound to the indicated GST-fusion proteins are shown. Gels were run in parallel, blotted to polyvinylidene difluoride membranes, and probed with anti-Ret (*left*) or anti-phosphotyrosine antibodies (*right*).

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 PLCγ2 (17); and 131 residues with 95% identity to the C-terminal 131 residues of human Enigma containing all of LIM2 and LIM3 (13).

β-Galactosidase Assay—Two hybrid transformants were assayed for β-galactosidase activity by solution assay (18). Units of activity were calculated as: activity = $1750(A_{420})/((\text{time in min})(\text{volume of culture in assay})(A_{600}\text{ of culture}))$.

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^{α}-*p*-tosyl-L-lysine chloromethyl ketone, 1 mM phenylmethylsulfonyl fluoride, 1 mM $NaVO_4$). Cleared lysates were incubated for 2 h with 2 μg of GST-fusion protein bound to glutathione-agarose beads in a total volume of 300 μl. 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-PLCγ, murine PLCγ SH2 domain 1 obtained from the two-hybrid screen; GST-Grb10, murine Grb10 SH2 domain obtained from the two-hybrid screen.

Microinjection Mitogenic Activity Assay—The development of this assay is described in detail elsewhere (11). Briefly, mouse 10T1/2 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 μg/ml Ret/ptc2 expression plasmid DNA and 8 mg/ml rabbit IgG (Sigma). For co-injection experiments, 200 μg/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, Tujunga, 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.

RESULTS

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 PLCγ, and a C-terminal fragment of Enigma that contained LIM domains 2 and 3 (LIM2/3). The interaction of Ret with PLCγ 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 β-galactosidase activity (Fig. 1*b*). 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,

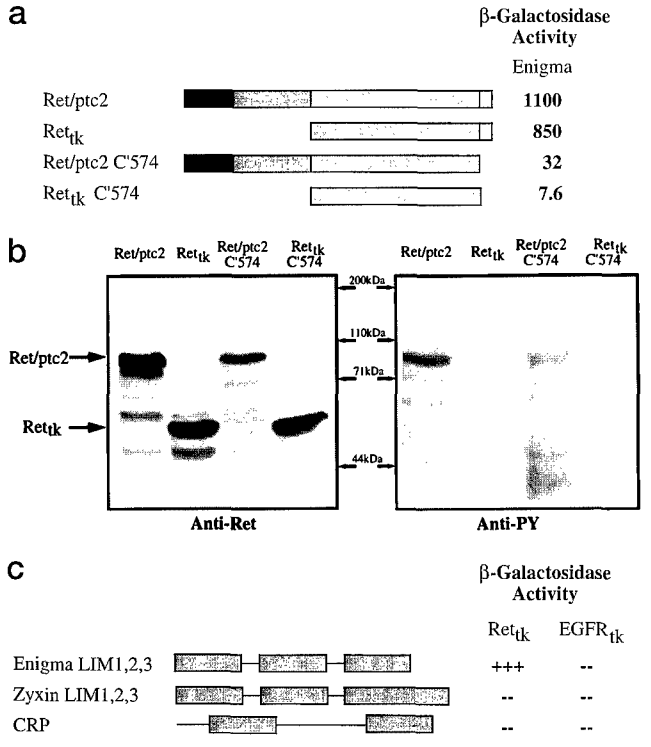

FIG. 2. **Requirements for the association between Ret/ptc2 and Enigma.** *a*, mapping of binding determinants in Ret/ptc2. Various fragments of Ret/ptc2, shown schematically, were used in the yeast two-hybrid system to measure interaction with the product of a plasmid expressing full-length Enigma. 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. 1*c*. *Anti-PY*, anti-phosphotyrosine. *c*, specificity of the LIM domains of Enigma for Ret/ptc2. pJG4–5 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$_{tk}$, the pEG202 plasmid expressing the Ret kinase with an intact C terminus, or EGFR$_{tk}$, a pEG202 construct containing the intracellular domain of EGFR. β-Galactosidase activity was assessed by streaking transformants on 5-bromo-4-chloro-3-indoyl β-D-galactoside plates. +++, denotes dark blue after 12 h of incubation compared with white (− −) that was equivalent to background after 24 h.

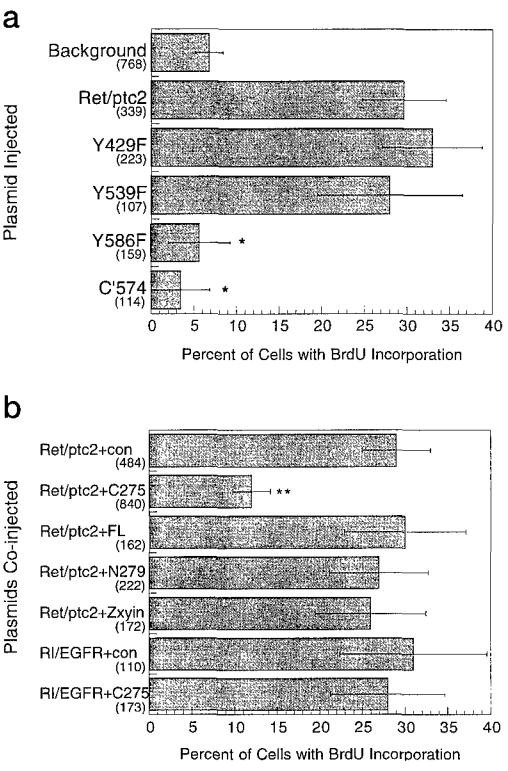

FIG. 3. **Effect of mutations in Ret that disrupt association with Enigma and of co-expression with the LIM domains of Enigma on mitogenic activity of RET/ptc2.** *a*, effects of mutations in Ret/ptc2. Serum-starved mouse fibroblasts (10T1/2) were microinjected with plasmids expressing either wild-type Ret/ptc2 or various mutants and then assessed for entry into S-phase by immunofluorescent detection of 5-bromodeoxyuridine (*BrdU*) incorporation. The fraction of injected cells positive for 5-bromodeoxyuridine incorporation is shown with *error bars* displaying the 95% confidence interval calculated using the standard error of proportion. The *numbers* in *parentheses* are the total number of injected cells. Plasmids were injected at a concentration of 100 μg/ml. *Asterisk* denotes a highly significant difference between cells injected with Ret/ptc2 or Y586F and Ret/ptc2 or C′574 ($p < 0.001$). *b*, effects of co-expression of Enigma and its fragments. Serum-starved fibroblasts were microinjected with a mixture of two of the following expression plasmids: Ret/ptc2 or RI/EGFR, a construct analogous to Ret/ptc2 with the EGFR intracellular domain in place of the Ret kinase, plus either *con*, control empty plasmid; *C275*, the C-terminal 275 residues of Enigma containing LIM domains 1, 2, and 3; *FL*, full-length Enigma; *N279*, N-terminal 279 residues of Enigma lacking LIM domains; or *Zyxin*, LIM domains from Zyxin, residues 339–452. In each case Ret/ptc2 and RI/EGFR constructs were injected at 100 μg/ml, while the other constructs were present at 200 μg/ml. *Double asterisk* denotes a highly significant difference between cells injected with Ret/ptc2+con or Ret/ptc2+C275 ($p < 0.001$).

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 (C′574).

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 NIH3T3 cells expressing the EGFR/Ret chimeric receptor (Fig. 1*a*), where EGF-dependent activation of EGFR/Ret has been characterized (12). In each case binding to EGFR/Ret from lysates of EGF-treated cells was observed (Fig. 1*c*). 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 EGF-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-phosphoty-

[2] Wu, R.-Y., Durick, R., Songyang, Z., Cantley, L. C., Taylor, S. S., and Gill, G. N. (1996) *J. Biol. Chem.*, in press.

rosine antibodies. Enigma bound to both Ret/ptc2 and to the Ret tyrosine kinase alone (Ret_{tk}) but did not bind to either when the C-terminal 23 residues were deleted (C'574, Fig. 2*a*). The interaction was not dependent upon the phosphorylation state of Ret because Ret_{tk} was not phosphorylated on tyrosine in the absence of the dimerization domain of RI, whereas Ret/ptc2 underwent tyrosine autophosphorylation (Fig. 2*b*, *lanes* Ret_{tk} and *Ret/ptc2*).

Based on optical densitometry, the extent of tyrosine phosphorylation of Ret/ptc2 and the C-terminal deletion Ret/ptc2 C'574 were equivalent. Because deletion of the C terminus of Ret/ptc2 did not decrease the phosphotyrosine content by a detectable amount, Tyr-586, the only tyrosine in the C terminus of this isoform of Ret, does not appear to be a major site of autophosphorylation. Results using both the EGFR/Ret chimera and Ret/ptc2 thus indicated that interaction with Enigma required the C terminus containing Tyr-586 but was independent of tyrosine autophosphorylation of Ret.

The interaction between LIM2 of Enigma and Ret was specific because LIM domains from other proteins failed to interact with Ret (Fig. 2*c*). Another tyrosine kinase, EGFR, failed to interact with the LIM domains of Enigma either in the two-hybrid system (Fig. 2*c*) or in GST-fusion binding reactions (data not shown).

Effect of Enigma on Ret/ptc2 Mitogenic Activity—The functional significance of the association between Ret and Enigma was investigated *in vivo* using microinjection. Various Ret/ptc2 expression constructs were injected into nuclei of serum-starved fibroblasts, and the capacity of these constructs to induce DNA synthesis was assayed by monitoring incorporation of the thymidine analog 5-bromodeoxyuridine. Mutations in Ret/ptc2 that blocked association with Grb10 (Y429F) or PLCγ (Y539F) had no significant effect on mitogenic activity, while mutation of Tyr-586 to Phe or deletion of the C terminus of Ret/ptc2 completely blocked the ability of Ret/ptc2 to induce DNA synthesis (Fig. 3*a*).

The strong correlation between mutants that failed to bind Enigma and loss of mitogenic activity suggested that Enigma was either required for the mitogenic signaling of Ret/ptc2 or that some other protein, which was not detected in the two-hybrid screen, also binds at Tyr-586. To discriminate between these two possibilities, co-injection experiments were performed to attempt to block the Ret/ptc2 mitogenic signal. Co-injection of Ret/ptc2 with a plasmid that expressed the three LIM domains of Enigma (C275, Fig. 3*b*) blocked Ret-induced DNA synthesis, while co-injection with full-length Enigma had no effect. These results support the conclusion that Ret/ptc2 requires Enigma for mitogenic signaling. If Enigma was simply competing for binding with some other signaling protein, then both full-length Enigma and the LIM domains alone should block signaling, given that both interact with Ret with an equivalent affinity.[2] The inhibition of mitogenesis was specific to the LIM domains of Enigma because the LIM domains of Zyxin (23) were without effect. It was also specific for Ret because the LIM domains of Enigma did not block the mitogenic activity of the EGFR tyrosine kinase analog of Ret/ptc2 (RI/EGFR) previously shown to have mitogenic activity in this assay (11).

DISCUSSION

LIM domains contain approximately 50 amino acids, bind two atoms of Zn^{2+}, 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-586-based motif in Ret.[2] 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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