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(CX2C)3-CX3-HX-CX5-CX5-CX5 (1). They bind two atoms of Zn$^{2+}$ with coordination being $S_4$, $S_2N_2S_2O_2$, or $S_2N_2O_2$ (2). The NMR structure of the carboxyl-terminal LIM domain of chicken cysteine-rich protein (CRP) revealed that the two Zn$^{2+}$ binding modules are located at the ends of a hydrophobic core composed of antiparallel $\beta$ 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-Phen 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, was found to interact with two receptor tyrosine kinases, determining the molecular basis of this recognition became important. We found that LIM2 of Enigma
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 Lichteiner, 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 Xim-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 Nazionale 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 525-nm emission anti-EGFR antibody was used to isolate a subline expressing high levels of this chimeric receptor. The murine anti-InsR antibody was purchased from BAbCO, Berkeley, CA; and the anti-phosphotyrosine antibody PY-20 was from Transduction Laboratories (Lexington, KY). A rabbit polyclonal 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-Ile-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 cRet, 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 280 and an extinction of ~3400 mV cm⁻¹) were dried in a speed vac (Savant), resuspended in 20 mM Tris-HCl, pH 7.4, and refiltered. To avoid problems with sequencing and oxidation, the total thespectrum was acidified for a medium-yellow color and immediately diluted 1:10 or 1:20 with buffer Z and permeabilized by SDS and chloroform. o-Nitrophenyl β-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 \((A_{600}/A_{590}) \cdot v\) when \(t = \text{time}\) and \(v = \text{volume}\) (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, 100 mM glyceral, 150 mM NaCl, 5 mM KCl, 1 mM EDTA, 2 mM phenylmethylsulfonyl fluoride, 1 mM Na₃VO₄, 10 mM benzamidine, 10 μg/ml of aprotonin 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). Enzyme-competent Ret/ptc2 and wild-type 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 Tyr-based peptide library comprising peptides of the sequence: Met-Ala-X-X-X-Tyr-X-X-X-X-Ala-Lys-Lys-Lys, where X indicates any 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 15². 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 microsequencing. 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. 1A. 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-Pro-X-Pro-X-Pro-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 18².
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. 1B). In addition, a Tyr residue was highly selected at position 14. 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. 1C). 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 holoInsR. 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-Ser-Ser-Ala-Leu-Gly-Ala-Leu-Tyr-Ala-Ser-Ser-Ala). 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.

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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 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 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 specificity of interaction of Ret/ptc2 and InsR with other LIM domains was further examined. As shown in Fig. 3B, 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 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-C9/Ret) and tested for their ability to bind Enigma. GST and GST-C9/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. 4A). Equal amounts of GST and GST-C9/Ret were assessed for their ability to bind these Enigma proteins (Fig. 4B). GST-C9/Ret bound full-length Enigma and

D. Winge, personal communication.
residues abolished interaction but a Phe substitution for Tyr in the LIM2 domain of Enigma. Mutation of the Asn and Tyr in the carboxyl terminus of Ret is necessary for interaction with these results indicate that the sequence peptide corresponding to exon 16/InsR were without effect. Ret, while the Ret mutant peptide Leu-Tyr, and mutant peptide, Asn-Lys-Leu-Phe, showed in Fig. 5B, incubation with wild type peptide, Asn-Lys-Leu-Tyr and Asn-Lys-Leu-Phe displaced EGFR/Ret binding to LIM2/Enigma (Fig. 5C). 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 Tyr586 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 Tyr586 with Phe in Ret/ptc2 on Enigma binding were quantitated. Wild-type Ret/ptc2 (Tyr586) and mutant Y586F Ret/ptc2 (Phe586) were expressed in 293 cells and the relative affinities of these proteins for the LIM domains of Enigma were measured. As shown in Fig. 5D the affinity of Tyr586 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 chimera 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. 6A). EGFR 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. 6B, 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).
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-Enigma 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. 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 pGal-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.

**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.
**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 Tyr586 with Y586F Ret/ptc2 to the carboxyl terminus of Enigma. Wild-type Ret/ptc2 (Tyr586) and the point mutation Y586F Ret/ptc2 (Phe586) 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.).
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 processes requiring the target sequences in InsR and Ret (25). The ability of LIM3 of Enigma to recognize the major endocytic code 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, Tyr1062 in holo Ret corresponding to Tyr586 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 Enigma 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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**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 chimera receptor utilized EGFR 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.
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