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Proliferation of breast cancer cells is controlled by interaction of peptide growth factors with their cell surface receptors. The major attenuation mechanism for signaling involves ligand-induced internalization and down regulation of receptors, a process dependent on both intrinsic tyrosine kinase activity and specific endocytic codes. To determine mechanisms through which cells recognize this information, we utilized exon 16 of insulin receptor (InsR) in the two-hybrid system to isolate a novel protein, Enigma which contains three LIM domains within its carboxyl terminus. Mutational analyses indicated that the carboxyl terminal LIM domain of Enigma specifically recognized active but not inactive endocytic codes in InsR. Enigma failed to interact with the fragments of IGF1-R, LDLR, Tfr and EGFR that contain endocytic codes. Further specificity was indicated by failure of other LIM domains to interact with exon 16 of InsR. Interestingly LIM2 of Enigma specifically recognized a distinct receptor tyrosine kinase, the endocytic code of IGF1-R was recognized by a LIM domain of Zyxin. We conclude that LIM domains recognize proteins through interaction with an exposed tyrosine located in tight turns. Variations in the sequence of tight turns and of LIM domains provide specificity for protein-protein interactions.

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FOREWORD

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Introduction:

Proliferation of normal and malignant breast cancer cells is controlled by interaction of peptide growth factors with their cognate cell surface receptors. The major attenuation mechanism for signaling involves ligand-induced internalization and down regulation of receptors, a process dependent on both intrinsic tyrosine kinase activity and specific endocytic code motifs (1). When receptors are excessively expressed, the endocytic mechanism is saturated and, because attenuation can not occur, excessive signaling ensues. Identification of the cellular machinery which recognizes the specific endocytic codes of ligand-activated tyrosine kinase receptors can provide a novel approach to control signaling by these receptors.

Ligand-induced internalization and down regulation depend upon well-defined sequence motifs; these are particularly well defined in the insulin receptor (InsR) and the epidermal growth receptor (EGFR). Two internalization codes, GPLY and NPEY, have been identified in exon 16 located in the juxtamembrane region of the cytoplasmic domain of the InsR (2) and peptides corresponding to both sequences adopt a tight turn structure in solution (3, 4). Mutation of GPLY to APLA and NPEY to APEA reduced InsR internalization to 32 and 87%, respectively, of the rate of wild-type InsR and mutation of both codes abolished ligand-induced internalization (2). A tyrosine-containing tight turn structure is the essential recognition motif present in endocytic codes of many receptors.

With identification of the endocytic codes has come an opportunity to use molecular recognition as a criterion for isolating specific proteins and subsequently defining the complex machinery involved in receptor trafficking. As the first step towards understanding the mechanisms of the internalization of InsR, we have sought to isolate a novel protein that binds specifically to the functionally defined endocytic codes of human InsR by the two-hybrid system (5). This 455 amino acid protein contains three tandemly arrayed LIM domains at its carboxyl terminus.

LIM (*C. elegans* Lin-11 (6), rat Isl-1 (7), and *C. elegans* Mec-3 (8)) was initially identified as a Cys-rich domain located at the NH₂ terminus of homeodomain proteins involved in development. LIM domains have also been found in zyxin and cCRP that are associated with focal adhesions

and cytoskeleton (9, 10). LIM domains, which bind Zn^{2+} (11, 12) and are often present in more than one copy within proteins, are proposed to function in protein-protein interactions both in homeodomain proteins that act in nucleus and in proteins that act in the cytoplasm and with the cytoskeleton.

Result:

Using the two-hybrid reagents developed by Roger Brent and colleagues (13), a 500-base pair HeLa cDNA was identified that encoded a 7-kDa protein fragment that interacted with the 22 amino acids of exon 16. This cDNA fragment was used to isolate a full length cDNA from a lgt11 cDNA library prepared from SK-N-MC cells. The cDNA contains an open reading frame encoding a 455 amino acid protein. The cDNA corresponding to a single mRNA species of 1.7 kilobases determined by Northern blotting, contains a Kozak sequence at the initiator Met, and the reading frame confirms that determined for the carboxyl terminal 7-kDa fragment produced as a fusion protein by plasmid pJG4-5 in the two hybrid screen. In vitro translation yielded a 55 kDa protein as expected from the open reading frame. Moreover, when full length enigma was placed into pJG4-5 it specifically interacted with exon 16 in the two hybrid system. Western blotting with an affinity purified antibody generated against the 7-kDa fragment confirmed a single cytoplasmic ~55-kDa protein in SK-N-MC and HeLa cells in agreement with the open reading frame and in vitro translation.

Although Enigma is a novel protein, the 7-kDa carboxyl terminus that interacted with exon 16 in the two-hybrid system consists predominantly of a LIM domain. Enigma contains three LIM domains in its carboxyl terminus. Holo-InsR was bound to the 7-kDa COOH terminus of Enigma expressed as a GST fusion protein but not to GST alone. To determine whether enigma recognized the active endocytic codes of InsR, the two-hybrid system was used to measure interaction of Enigma with mutant exon 16 sequences. Both the 7-kDa COOH-terminal fragment containing LIM3 and holo-Enigma were used. A 2-amino acid change in the strong endocytic code (GPLY to APLA) almost completely abolished interaction. A 2 amino acid change in the weaker endocytic code (NPEY to APEA) decreased interaction but did not abolish it. No interaction

occurred when both mutations were present. There is thus strong concordance between effects of these point mutants in InsR in vivo (2, 4) and in vitro interactions between Enigma and exon 16.

Although many receptors internalize via coated pits, specificity is implied by observations that EGFR do not compete with transferrin receptor for internalization (14) nor do InsR compete with IGF-II/mannose 6-phosphate receptors for internalization (15). To investigate specificity, we tested the interaction of LIM3 and holo-Enigma with endocytic codes from other receptors. No interaction was detected using the two-hybrid system with the cytoplasmic domains that contain the endocytic codes of IGF1-R, LDLR, TfR, or EGFR. Interaction between LIM3 of Enigma and the GPLY strong endocytic code of InsR thus showed high specificity.

Specificity for LIM3 recognition of exon 16 was examined using more LIM domains from Enigma, Mec-3, Isl-1, Xlim1, CRP and Zyxin. LIM domains were cloned into pJG4-5 in frame and co-transformed with individual endocytic code into yeast. None of the tested LIM domains except LIM3 of Enigma recognized exon 16. The specificity of Enigma for exon 16 thus resides in LIM3. Interestingly enough, LIM2 of Zyxin specifically recognized the endocytic codes of IGF1-R. Deletion of the strong endocytic code NPEY abolished the interaction.

Specificity of LIM domain recognition was further studied using degenerate peptide library selections and peptide competitions. Interaction of two random peptide libraries with GST-LIM3 of Enigma indicated specific binding of the G P Hydrophobic G P Hydrophobic Y A sequences of exon 16 of InsR. A immobilized GST-LIM3 of Enigma fusion protein bound to holo InsR; a synthetic peptide corresponding to exon 16 of InsR displaced InsR with a K_i of ~20 μ M. LIM3 of Enigma did not bind exon 16 of IGF1-R nor did a synthetic peptide corresponding to exon 16 of IGF1-R displace InsR from GST-LIM3 of Enigma.

Conclusion:

The ability to specifically recognize the active endocytic code of InsR via a LIM domain fulfills the first property of the endocytic mechanism predicted from study of kinetics of ligand-induced saturable high affinity endocytosis of receptor, that of recognition, but additional criteria are necessary to demonstrate function in this process. Enigma recognized the

endocytic codes of exon 16 of InsR proportional to the strength of the codes in holo-InsR *in vivo*, but failed to recognize other endocytic codes. Specificity resided not only in the tyrosine-containing tight turn but in the LIM domain as shown by the inability of LIM1 of Enigma and both LIM1 and LIM2 of Mec-3 to interact with exon 16. Although these observations indicate specificity for both partners to the interaction, they do not prove a function for Enigma which may act in ways unrelated to insulin action. The two-hybrid system has high sensitivity for detecting protein interaction, and it remains possible that physiological relevant interactions with exon 16 and with Enigma will be quite different from those described. The interaction between exon 16 of InsR and LIM3 of Enigma do nonetheless provide evidence for a novel protein interaction mechanism. We conclude that LIM domains are a protein dimerization motif with specificity residing in both the LIM domain and target. A tyrosine-containing tight turn and the flanking sequences of exon 16 of InsR represents one class of specific targets for a LIM domain.

Plans for Coming Year:

We have discovered that LIM 2 of Enigma specifically recognizes a distinct receptor tyrosine kinase (RTK). None of other LIM domains interact with this RTK. This is further evidence for specificity. This RTK was recently identified as the gene responsible for several human syndromes. Further objectives for coming-year include:

- 1). Identification of interaction site in RTK using two-hybrid system and GST-fusion for *in vitro* interaction.
- 2). Co-immunoprecipitation of RTK and Enigma for *in vivo* interaction.
- 3). Mutation of recognition domain in RTK to define function of Enigma in signal transduction and trafficking by assay *in vivo*.

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Publications and Meeting Abstracts as a Result of Contract Support:

Publications:

[1] **R. Wu** and G. Gill, "LIM Domain Recognition of a Tyrosine-containing Tight Turn", J. Biol. Chem., 1994, vol. 269, pp. 25085-25890

[2] C. Lazar, **R. Wu** and G. Gill, "Basolateral Sorting of Epidermal Growth Factor Receptors in MDCK Cells Requires Sequences Located in the Regulatory Carboxyl Terminus", submitted to Molecular Biology of the Cell.

Abstracts:

[1] **R. Wu** and G. Gill, "LIM Domain Recognition of Tyrosine-containing Tight Turn", American Society for Cell Biology Annual Meeting, San Francisco, Dec. 1994

[2] C. Lazar, **R. Wu** and G. Gill, "Basolateral Sorting of Epidermal Growth Factor Receptors in MDCK Cells Requires Sequences Located in the Regulatory Carboxyl Terminus", American Society for Cell Biology Annual Meeting, San Francisco, Dec. 1994

[3] **R. Wu** and G. Gill, "Differential Recognition of Endocytic Regions of the Insulin and Insulin-Like Growth Factor-1 Receptors by Specific LIM Domains", American Society for Clinical Investigation Annual Meeting, San Diego, May 1995