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Insulin Receptor Substrate-2 Binds to the Insulin Receptor through Its Phosphotyrosine-binding Domain and through a Newly Identified Domain Comprising Amino Acids 591–786*

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We compared the interaction between the insulin receptor (IR) and the IR substrate (IRS) proteins (IRS-1 and IRS-2) using the yeast two-hybrid system. Both IRS proteins interact specifically with the cytoplasmic portion of the IR and the related insulin-like growth factor-I receptor, and these interactions require receptor tyrosine kinase activity. Alignment of IRS-1 and IRS-2 revealed two conserved domains at the NH_2 terminus, called $IH1^{PH}$ and $IH2^{PTB}$, which resemble a pleckstrin homology (PH) domain and a phosphotyrosine binding (PTB) domain, respectively. The $IH2^{PTB}$ binds to the phosphorylated NP*X*Y motif (Tyr-960) in the activated insulin receptor, providing a specific mechanism for the interaction between the receptor and IRS-1. Although the $IH2^{PTB}$ of IRS-2 also interacts with the NPEY motif of the insulin receptor, it is not essential for the interaction between the insulin receptor and IRS-2 in the yeast two-hybrid system. IRS-2 contains another interaction domain between residues 591 and 786, which is absent in IRS-1. This IRS-2-specific domain is independent of the $IH2^{PTB}$ and does not require the NPEY motif; however, it requires a functional insulin receptor kinase and the presence of three tyrosine phosphorylation sites in the regulatory loop (Tyr-1146, Tyr-1150, and Tyr-1151). Importantly, this novel domain mediates the association between IRS-2 and insulin receptor lacking the NP*X*Y motif and may provide a mechanism by which the stoichiometry of regulatory loop autophosphorylation enhances IRS-2 phosphorylation.

IRS[1] proteins (IRS-1 and IRS-2) are substrates for the insulin and IGF-I (insulin-like growth factor-I) receptors and tyrosine kinases associated with the receptors for various cytokines, including growth hormone, several interleukins (IL-2, IL-4, IL-9, IL-13, and IL-15), and interferons (interferon α/β, and interferon γ) (1–9). IRS proteins provide interfaces between these activated receptors and various signaling proteins, especially those with Src homology-2 domains (SH2 proteins). During insulin stimulation, IRS-1 associates with several signaling proteins, including the regulatory subunits of the phosphatidylinositol 3-kinase (p85α/β and $p55^{PIK}$), SH-PTP2, Nck, Grb-2, and Fyn (10–13). The use of IRS proteins rather than receptor autophosphorylation sites to engage signaling molecules provides a mechanism to dissociate the signaling complex from the activated receptor and amplify the signal. However, the use of IRS proteins requires a specific mechanism to select the appropriate activated receptor.

Previous reports suggested that Tyr-960 in the NPEY motif of the insulin receptor juxtamembrane region is essential for insulin signal transmission and substrate phosphorylation (14, 15). A similar NP*X*Y motif in the IL-4 receptor is also required for IRS-1 phosphorylation (16). The recent identification of the phosphotyrosine binding (PTB) domain in the IRS proteins ($IH2^{PTB}$), which binds to phosphorylated NP*X*Y motifs, provides a provisional explanation for interaction between IRS proteins and the receptor for insulin, IGF-I, and IL-4 (17–21). In addition, the pleckstrin homology domain at the NH_2 terminus of IRS-1 ($IH1^{PH}$) also contributes to its interaction with the insulin receptor; however, the mechanism of this interaction is unknown (22).

Since many receptors that mediate IRS protein phosphorylation do not contain NP*X*Y motifs, the $IH1^{PH}$ domain or some other mechanism must be involved. To search more precisely for other regions of interaction between IRS proteins and the insulin receptor, we have employed the yeast two-hybrid system. Our results reveal an novel region in IRS-2 that interacts with the kinase domain of the insulin receptor. Unlike the $IH2^{PTB}$ domain, this region interacts with tyrosine residues in the regulatory loop of the kinase domain. Since an equivalent region was not detected in IRS-1, our results reveal an important difference between IRS-1 and IRS-2, which may play a role in signaling specificity between those two IRS proteins.

EXPERIMENTAL PROCEDURES

Materials—For the two-hybrid system we used the yeast strain L40 (MAT **a**, *trp1, leu2, his3, LYS2::lex A-HIS3, URA3::lex A-lacZ*) and the yeast expression plasmid pBTM116. Both reagents were provided by A. Vojtek (Seattle, WA). The yeast expression plasmid pACTII was provided by S. Elledge (Houston, TX). The L40 strain and the two-hybrid plasmids have been described earlier (23, 24). The human IR and IGF-IR cDNAs were obtained from A. Ullrich (Munich, Germany) and P. De Meyts (Copenhagen, Denmark), respectively.

Synthetic defined dropout yeast media lacking the appropriate amino acids were obtained from BIO 101 (La Jolla, CA). Oligonucleotides were purchased from Genset (Paris, France). All chemical reagents were from Sigma France, and enzymes were from New England Biolabs (Beverly, MA).

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[1] The abbreviations used are: IRS, insulin receptor substrate; IGF-I, insulin-like growth factor-1; IL, interleukin; IGF-IR, insulin-like growth factor-1 receptor; IR, insulin receptor; SH2 domain, Src homology-2 domain; PTB, phosphotyrosine binding domain; LDBD, LexA DNA binding domain; GAD, Gal4 activation domain; X-Gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; SC, synthetic complete.

Plasmid Construction—The coding sequences of the cytoplasmic domain of IR (amino acids 944-1343) and IGF-IR (amino acids 933-1337) were subcloned in the plasmid pBTM116 as described previously (19). This vector contains a Trp^+ selection marker. These fragments were in frame with the LexA DNA binding domain (LDBD), yielding LDBD-IRβ and LDBD-IGF-IRβ hybrid constructs, respectively. By site-directed mutagenesis of double strand DNA using the Transformer™ kit (Clontech, Palo Alto, CA), different mutations and deletions of the IR were obtained. These mutations were verified by DNA sequence analysis using the T7 sequencing kit (Pharmacia Biotech, Uppsala, Sweden).

The mouse IRS-2 cDNA amino acids 2–1324 were subcloned into the plasmid pACTII, encoding the GAL4 activation domain (GAD). The GAD-IRS-2 construct was generated by the use of convenient restriction sites maintaining the appropriate reading frame.

Several subdomains of IRS-2 were amplified by the polymerase chain reaction using the Pwo DNA polymerase (Boehringer Mannheim, France) with different sets of the following primers (5′ to 3′): cgcggatccgctaccgcgaggtgtggcag (1), ccggaattctgctgggggggtggccgc (2), cgcggatccgcctagatgaatacactctc (5), ccggaattcgaggccttcactgcctcc (6), ccggaattctcaaagggcctcacctttcacgac (2′), cgcggatccgcgccaagtgcacttcgtgccgg (7), and ccggaattcagaggcagaggaaggctgagg (8). *Eco*RI or *Bam*HI sites are underlined. The polymerase chain reaction products digested with *Eco*RI and *Bam*HI were cloned into pACTII (vector containing Leu^+ selection marker), digested with *Eco*RI and *Bam*HI. The inserted fragment was in frame with the activation domain of Gal4. The plasmids obtained by cloning the DNA fragment were amplified with the primer sets 1/2, 5/6, 5/2′, and 7/8; they encode GAD hybrid proteins with subdomains containing amino acids 191–350, amino acids 591–786, amino acids 591-1325, and amino acids 351–590, respectively.

Rat IRS-1 and different subdomains were cloned into the pACTII in the same fashion as IRS-2. The GAD-IRS-1 construct was described previously (19), and the fragment of IRS-1 was obtained by polymerase chain reaction using the following primers (5′ to 3′): cgcggatccgcgacttgagctatgacacgggc (1) and ccggaattctggtttcccacccaccatact (2). *Eco*RI or *Bam*HI sites are underlined. The plasmids encode GAD hybrid protein with the subdomain containing amino acids 144–316 with primer sets 1/2.

Transformation of Yeast Strains and β-Galactosidase Assay—The yeast strain L40 was transformed by the lithium acetate method of Gietz *et al.* (25) using different combinations of plasmids. Transformants were grown in synthetic complete (SC) plates lacking tryptophan and leucine to select for the presence of pBTM116 and pACTII, respectively. Individual transformants were patched on SC medium lacking tryptophan and leucine and then replicated on Whatman No. 40 paper laid on an SC medium without tryptophan and leucine. After 24 h, a β-galactosidase assay was performed by a color filter assay using 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) as described (26). A blue coloration of colonies reflects the occurrence of interaction with the two hybrid proteins. Cotransformants were also tested for histidine prototrophy after replica plating on SC medium without tryptophan, leucine, and histidine. For quantitative studies of the β-galactosidase activity, a liquid culture assay using *o*-nitrophenyl-β-D-galactopyranoside as a substrate was carried out according to Ref. 26.

RESULTS AND DISCUSSION

Full-length IRS-2 Interacts with IR and IGF-IR—We have studied the interaction of IR and IGF-IR (wild type and mutated forms) with IRS-2 by using the yeast two-hybrid system (27). In our approach, the cytoplasmic domain of IR and IGF-IR cDNAs were fused to the LDBD. Full-length or subdomains of IRS-2 cDNAs were fused to the activation domain of Gal4. These two hybrid proteins were coexpressed in L40 yeast strain containing *lacZ* and *HIS3* genes as reporter genes (23). Since the transcription of these genes is under the control of LexA upstream-activating sequences, when the two fused proteins associate, the reporter genes are transcribed, and cotransformants produce β-galactosidase and grow in the absence of histidine.

To establish whether IRS-2 binds to IRβ and IGF-IRβ, we tested the interaction of LDBD-IRβ and LDBD-IGF-IRβ with GAD-IRS-2. When IRS-2 was expressed with IRβ or IGF-IRβ, cotransformants were blue in the X-Gal assay (Fig. 1*A*). In contrast, when IRS-2 was coexpressed with lamin, the X-Gal assay was negative as expected. Moreover, no interaction occurred between GAD-IRS-2 and the kinase inactive receptor in which lysine 1018 was replaced with alanine (LDBD-IRβ K1018A) (Fig. 1*B*). Thus, we conclude that, like for IRS-1 (17), the interaction between IR and IRS-2 is dependent on tyrosine phosphorylation of the insulin receptor. The insulin receptor undergoes tyrosine autophosphorylation in three regions including the regulatory loop (Tyr-1146, Tyr-1150, Tyr-1151), the COOH terminus (Tyr-1316, Tyr-1322), and the juxtamembrane region (Tyr-960) (28).

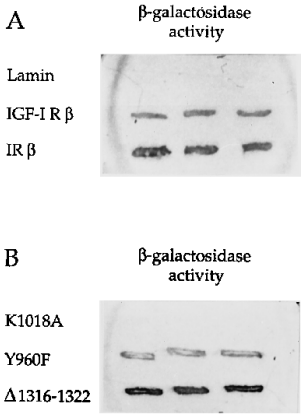

FIG. 1. **Characterization of the interaction between IR and IGF-IR with IRS-2.** The yeast reporter strain L40 was cotransformed with the plasmid encoding GAD-IRS-2 in combination with plasmids encoding various LDBD receptor constructs. After isolation on selective plates, transformants were assayed for β-galactosidase activity by performing a color filter assay as described under "Experimental Procedures." *A*, coexpression of GAD-IRS-2 with LDBD-IRβ (amino acids 944-1343) or LDBD-IGF-IRβ (amino acids 933-1337). As a negative control, the IRS-2 hybrid was coexpressed with the LDBD-lamin hybrid. *B*, coexpression of GAD-IRS-2 with LDBD-IRβ-mutated forms. K1018A contains the mutation of lysine 1018 to alanine. This mutation abolishes the ATP-binding site and the tyrosine kinase activity of IRβ. Y960F has the tyrosine 960 mutated to phenylalanine, and Δ1316–1322 is a IR mutated form with the amino acids 1316–1322 deleted. A *blue* color of the colonies (shown in *black*) indicates an interaction with the two hybrid proteins. Similar results were obtained by analyzing growth on SC plates lacking histidine.

We tested the requirement for tyrosines 1316 and 1322 of the IR in this association by coexpressing GAD-IRS-2 with LDBD-IRβ Δ1316–1322. This mutant IR lacked two autophosphorylation sites (tyrosines 1316 and 1322) because of the deletion of residues 1316–1322. This mutant receptor interacted normally with IRS-2, demonstrating that the tyrosine residues 1316 and 1322 do not mediate the interaction (Fig. 1*B*).

Since tyrosine 960 of the juxtamembrane domain of IR has been shown to correspond to a key binding site of IRS-1, we investigated whether this residue plays a role in the association with IRS-2. To do this we coexpressed GAD-IRS-2 with LDBD-IRβ Y960F, which contains phenylalanine in place of tyrosine 960. Surprisingly, this mutation did not prevent the interaction between the LDBD-IRβ and the full-length GAD-IRS-2 (Fig. 1*B*). These results were unexpected because the $IH2^{PTB}$ domain in IRS-2 binds specifically to phosphorylated NP*X*Y motifs with similar characteristics to those of IRS-1.[2] Furthermore, a GAD hybrid protein containing the $IH2^{PTB}$ domain from IRS-2 and IRS-1 interacted specifically with the wild type IR but not with the IR Y960F (Fig. 2). Thus, the interaction of IRS-2 and IRS-1 with the IR involves their PTB domain, and both IRS proteins seem to interact in a similar fashion. However, our finding that IRS-2 still associates with IR Y960F suggests that another domain of IRS-2 interacts with a cytoplasmic region of the IR, which is different from the juxtamembrane NP*X*Y motif.

[2] L. Yenush and M. F. White, manuscript in preparation.

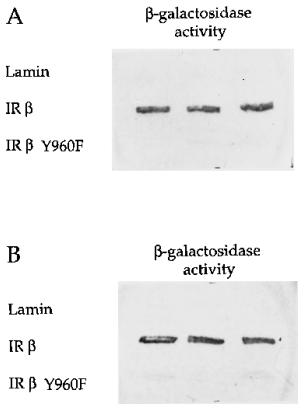

FIG. 2. **Interaction between the isolated PTB domain of IRS-2 and IRS-1 with the IR tyrosine 960.** The yeast reporter strain L40 was cotransformed with the plasmids encoding the LDBD fused to either IRβ (amino acids 944-1343) or IRβ mutated on tyrosine 960 (IRβ Y960F) in combination with plasmids encoding GAD fused to the isolated PTB domain of IRS-2 (GAD-PTB) (*A*) or isolated PTB domain of IRS-1 (GAD-PTB) (*B*). As a negative control, the IRS-1 and IRS-2 GAD-PTB constructs were coexpressed with the LDBD-lamin hybrid. Transformants were isolated on selective plates. Activation of the reporter gene *lexA-lacZ* was monitored by measuring the β-galactosidase activity in a color filter assay with X-Gal as substrate. A *blue* color (shown in *black*) indicates an interaction with the two protein hybrids. Similar results were obtained by analyzing growth on SC plates lacking histidine.

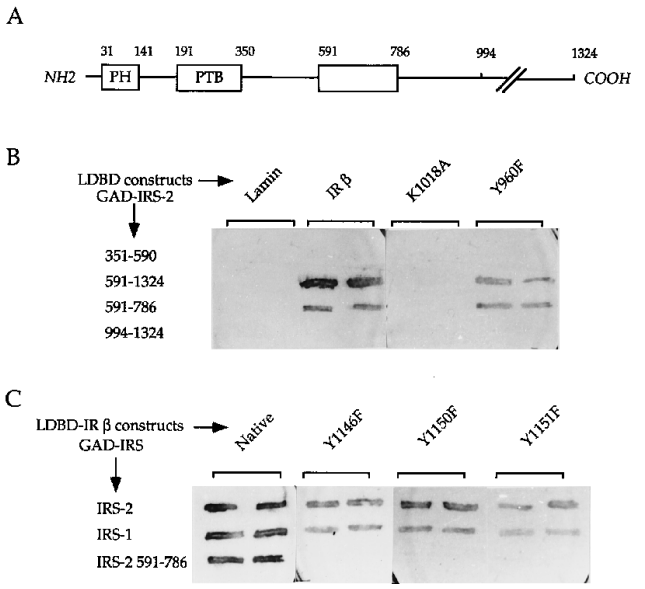

FIG. 3. **Characterization of the interaction of IR with the second interaction domain of IRS-2.** *A*, schematic representation of the mouse IRS-2 protein. *PH*, pleckstrin homology domain. Amino acids boundaries for each regions are indicated. *B*, fragments of IRS-2 corresponding to the indicated residues were obtained and expressed as fusion proteins with GAD. These GAD-IRS-2 constructs were cotransformed in the reporter strain L40 with plasmids encoding the indicated LDBD construct. The β-galactosidase activities in cotransformants were measured by the filter color assay using the substrate X-Gal. As a negative control, the IRS-2 hybrid constructs were coexpressed with the LDBD-lamin hybrid. *C*, the second interaction domain of IRS-2 (amino acids 591–786), IRS-2, and IRS-1 fused to the GAD were coexpressed with native LDBD-IR or the indicated mutated forms of LDBD-IR. Y1146F, Y1150F, and Y1151F have the Tyr-1146, Tyr-1150, and Tyr-1151 mutated to phenylalanine, respectively. The β-galactosidase activities of cotransformants were measured as indicated above. A *blue* color (shown in *black*) indicates an interaction between the two hybrid proteins. Similar results were obtained by analyzing growth on SC plates lacking histidine.

Identification of a Second Receptor Interaction Domain of IRS-2—To identify the novel interaction domain of IRS-2, which is distinct from the PTB, we examined the interaction between LDBD-IRβ and different deletion hybrids of IRS-2. Unlike IRS-1, the COOH terminus of IRS-2 (residues 591-1325) interacts specifically with the IR. The existence of such a domain may explain why intact IRS-2 bound to the Y960F IR construct. To localize more precisely this newly identified domain, we made two constructs: (i) GAD-IRS-2 containing amino acids 591–786; and (ii) GAD-IRS-2 containing amino acids 994-1324. The region of IRS-2 comprising amino acids 591–786 interacted with the IR, whereas the construct containing amino acids 994-1324 did not (Fig. 3*B*). Moreover, co-expression of these IRS-2 regions with LDBD-IRβ K1018A and LDBD-IRβ Y960F revealed that the interaction of IRS-$2^{591-786}$ required IR kinase activity but was independent of Tyr-960 (Fig. 3*B*).

The Tyrosine Autophosphorylation Sites in the Regulatory Loop IR Kinase Are Involved in Binding IRS-2—Finally, we investigated whether the interaction between IRS-$2^{591-786}$ and IR required the autophosphorylation sites in the regulatory loop (Tyr-1146, -1150, and -1151). To address this issue we replaced individually Tyr-1146, Tyr-1150, and Tyr-1151 with phenylalanine in the LDBD-IRβ. We found a reduced but persistent interaction of the different constructs of IR with GAD-IRS-1 and GAD-IRS-2 (Fig. 3*C*). These results indicate that the autophosphorylation sites in the regulatory loop regulated the association of IR with both IRS proteins. These data are similar to our observations made with IRS-1 and the IGF-IR (19). Next, we coexpressed the different autophosphorylation tyrosine mutants with the IRS-$2^{591-786}$ regions (Fig. 3*C*). Each point mutation completely inhibited the interaction. However, the corresponding region of IRS-1 (494–746) did not bind to wild type IR (data not shown). Thus, the tyrosine autophosphorylation sites Tyr-1146, Tyr-1150, and Tyr-1151 in the IR kinase domain play an active role in the regulation of the interaction with IRS-2 by associating directly with IRS-$2^{591-786}$.

The Second Domain of IRS-2 Interacts More Efficiently with the IR than the PTB Domain—To gain insight into the role of the two known IRS-2 domains in the interaction with IR, we coexpressed LDBD-IRβ with GAD-IRS-2, GAD-$IH2^{PTB}$-(191–350), or GAD-IRS-2-(591–786). We quantitated the interaction by measuring the β-galactosidase activity using a liquid test (Fig. 4*A*). We found that the level of the β-galactosidase activity, obtained when the native IRS-2 construct is expressed with the IR, was additive to that obtained when the receptor was expressed with the two domains separately. Interestingly, we observed that the IRS-2-(591–786) domain exhibited a 5-fold higher β-galactosidase activity than the PTB domain when coexpressed with the IR. These data suggest that the domain of IRS-2 comprising amino acids 591–786 interacts with the IR in a tighter fashion compared with the $IH2^{PTB}$ domain.

Finally, to investigate whether for IRS-2, the PTB domain and the newly identified domain 591–786 are the only two regions through which binding to the insulin receptor takes place, we constructed an IR mutated on both Tyr-1150 and/or Tyr-1151, and on Tyr-960. Co-expression of these mutants with GAD-IRS-2 indicates that the interaction between IR and IRS-2 is entirely lost (Fig. 4*B*). To verify the expression of IR mutants in yeast, we coexpressed the LDBD-IRβ Y960F/Y1150F and LDBD-IRβ Y960F/Y1150F/Y1151F with the GAD-p85 subunit of phosphatidylinositol 3-kinase. The transformation with p85 can be considered as a positive control because the SH2 domains of p85 interact with the COOH-terminal tyrosines 1316 and 1322 of the IR (29). Taking our results together, we conclude that IRS-2 interacts with the IR with two domains, *i.e.* the PTB domain and the domain comprising amino acids 591–786.

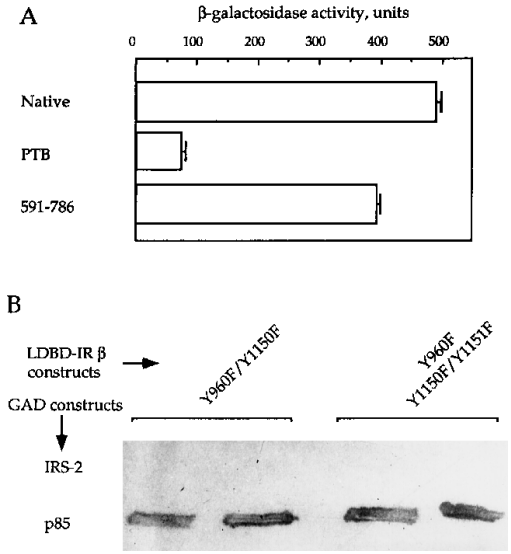

FIG. 4. **Contribution of the different IRS-2 domains in the interaction between IR and IRS-2.** *A*, the yeast reporter strain L40 was transformed with the plasmid encoding the LDBD-IRβ in combination with the plasmid encoding the GAD fused to IRS-2 native form (amino acids 2–1324), PTB domain of IRS-2 (amino acids 190–350), or IRS-2 (amino acids 591–786). The β-galactosidase activities in cell lysates were measured by a liquid test using the substrate *o*-nitrophenyl-β-D-galactopyranoside and were calculated according to Miller (30). Values represent the average ($\pm$S.E.) of six independent transformants. *B*, the yeast reporter strain L40 was transformed with the plasmid encoding the GAD fused to IRS-2 native or the GAD fused to p85 subunit with the indicated IR mutated forms, LDBD-IRβ Y960F/Y1150F and LDBD-IRβ Y960F/Y1150F/Y1151F, which are the tyrosines 960 and 1150 mutated to phenylalanine and tyrosines 960, 1150, and 1151 mutated to phenylalanine, respectively. The β-galactosidase activities of cotransformants were measured as indicated above. A *blue* color (shown in *black*) indicates an interaction between the two hybrid proteins. Similar results were obtained by analyzing growth on SC plates lacking histidine.

In summary, using the yeast two-hybrid system we have shown that IRS-2 binds to tyrosine-phosphorylated IR and IGF-IR. The analysis of IRS-2 binding to IR shows that IRS-2 associates via its PTB domain to the IRβ-subunit at the level of the NP*X*Y^{960} motif in the juxtamembrane domain. However, the key observation of our study is that IRS-2 interacts with the IR via a second domain comprising amino acids 591–786, which is different from the PTB domain. This newly identified domain associates with the catalytic domain of IR containing Tyr-1146, Tyr-1150, and Tyr-1151 residues. The IR interaction of IRS-2 through its PTB domain appears to be less tight compared with the interaction through the other IRS-2 domain (Fig. 4*A*). In addition, the interaction through the PTB domain does not abolish the binding of native IRS-2 to the IR (Fig. 1).

Taking these observations together we would like to suggest that the two domains might have different functions. Indeed, the IRS-2 domain comprising amino acids 591–786 would be the primary anchor of IRS-2 to the insulin receptor, while the PTB domain would have a stabilizing action on the interaction with the insulin receptor.

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