

# Tyr<sup>624</sup> and Tyr<sup>628</sup> in Insulin Receptor Substrate-2 Mediate Its Association with the Insulin Receptor\*

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**In addition to the pleckstrin homology domain and the phosphotyrosine binding domain in insulin receptor substrate (IRS)-1 and IRS-2, a region between amino acids 591 and 786 in IRS-2 (IRS-2-(591–786)) binds to the insulin receptor. Based on peptide competition studies, this region interacts with the phosphorylated regulatory loop of the insulin receptor; we designate this region the kinase regulatory loop binding (KRLB) domain. Two tyrosine residues in the KRLB domain at positions 624 and 628 are crucial for this interaction. Phosphorylation of tyrosine residues in the KRLB domain by the insulin receptor inhibits the binding to the receptor. These results reveal a novel mechanism regulating the interaction of the insulin receptor and IRS-2 that may distinguish the signal of IRS-2 from IRS-1.**

The insulin receptor (IR)<sup>1</sup> mediates tyrosine phosphorylation of several cellular substrates, including IRS-1, IRS-2, and Gab-1 (1–3). These IRS (insulin receptor substrate) proteins provide an interface between the activated insulin receptor and various signaling proteins. IRS proteins are composed of a COOH terminus containing multiple tyrosine phosphorylation sites in various amino acid sequence motifs that bind to the Src homology-2 domain in certain enzymes and adapter molecules (4–7). In addition to the phosphorylation sites, IRS proteins contain other domains to engage activated membrane receptors. At the extreme NH<sub>2</sub> terminus, the IRS proteins contain a pleckstrin homology (PH) domain (IH1<sup>PH</sup>). The IH1<sup>PH</sup> is essential in IRS-1 for the interaction with a physiological level of insulin receptor (8); this domain plays a similarly important role in IRS-2 and Gab-1. In addition to the PH domain, IRS-1 and IRS-2 contain a phosphotyrosine binding (PTB) domain

(IH2<sup>PTB</sup>), which binds to the phosphorylated NPEY motif in the cytoplasmic region of the receptors of insulin, insulin-like growth factor-I, and interleukin-4 (9–15).

A third region between residues 591 and 786 in IRS-2 engages the activated insulin receptor (13, 16). Using a yeast two-hybrid analysis, Tyr<sup>624</sup> and Tyr<sup>628</sup> in IRS-2 were found to contribute significantly to this interaction. The amino acid sequence in the region does not reveal a known protein-protein interaction domain, such as the PH domain, PTB domain, Src homology 2 domain, Src homology 3 domain, or WW domain (17–21). However, since it interacts with the phosphorylated regulatory loop of the insulin receptor  $\beta$ -subunit, we propose to designate it as the kinase regulatory loop binding (KRLB) domain. The binding of the KRLB domain of IRS-2 to the insulin receptor is independent of tyrosine phosphorylation sites in the COOH terminus and the NPEY motif in the juxtamembrane region (13). Since IRS-1 does not contain a KRLB domain (13, 16), this domain may contribute to a unique signaling potential of IRS-2.

## EXPERIMENTAL PROCEDURES

**Yeast Strains and Plasmids**—The yeast strain L40 (MAT a, *trp1*, *leu2*, *his3*, *LYS2::lexA-His3*, *URA3::lexA-lacZ*) and the yeast expression plasmids pBTM116 were obtained from A. Vojtek (Fred Hutchinson Cancer Research Center, Seattle, WA). The plasmid pACTII and the human insulin receptor cDNA were provided by S. Elledge (Baylor College of Medicine, Houston, TX) and A. Ullrich (Max-Planck-Institut für Biochemie, Martinsried, Germany), respectively. The two-hybrid plasmids and the L40 strains have been described previously (22, 23).

**cDNA Constructs**—Full-length mouse IRS-2 and IRS-2-(591–786) (IRS-2-KRLB) cDNAs were subcloned into the two-hybrid plasmid pACTII as reported previously (13). The subdomain of rat IRS-1 containing amino acids 494–741 was subcloned into pACTII. This fragment was obtained by polymerase chain reaction using the following primers (5' to 3'): *cgcggatccgcaccatggggacaaagccggcg* and *cgggaattcggggctgctggtgtggaatc*. *EcoRI* and *BamHI* sites are underlined. The polymerase chain reaction product digested with *EcoRI* and *BamHI* were cloned into pACTII digested with *EcoRI* and *BamHI*. All IRS-1 and IRS-2 point mutants were generated by either site-directed mutagenesis of double-stranded DNA using the Transformer<sup>TM</sup> kit (CLONTECH, Palo Alto, CA) or the Quickchange<sup>TM</sup> site-directed mutagenesis kit (Stratagene, San Diego, CA).

**Transformation of Yeast Strains and  $\beta$ -Galactosidase Assay**—Plasmid DNA transformations were performed using the lithium acetate method of Gietz *et al.* (24). Cotransformants were selected on *Trp*<sup>−</sup>, *Leu*<sup>−</sup> plates. The transformants were tested for  $\beta$ -galactosidase activity by liquid culture assays using the substrate *o*-nitrophenyl- $\beta$ -D-galactopyranoside (ONPG) as described by Miller (25).

**Construction of IRS-2 Fusion Proteins**—GST fusion proteins were generated by subcloning a fragment of IRS-2-KRLB containing the amino acids 591–786, either wild type or mutated, into the vector pGEX-3X (Pharmacia Biotech Inc., Uppsala, Sweden). All GST fusion proteins were expressed in BL21 bacterial cells. Bacteria were lysed in presence of lysis buffer (20 mM Tris, pH 7.4, 1 M NaCl, 0.2 mM EDTA, 0.2 mM EGTA) containing protease inhibitors and 1 mg/ml lysozyme. Lysates were frozen twice in liquid nitrogen and sonicated twice for 30 s. After 20 min of centrifugation at 17,000  $\times$  g, clarified lysates were

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<sup>1</sup> The abbreviations used are: IR, insulin receptor; IRS-1, insulin receptor substrate-1; IRS-2, insulin receptor substrate-2; KRLB, kinase regulatory loop binding domain; IRS-2-(591–786), amino acids 591–786 of IRS-2 containing the KRLB domain; PH, pleckstrin homology; IH1<sup>PH</sup>, IRS-homology 1 PH domain; PTB, phosphotyrosine binding domain; IH2<sup>PTB</sup>, IRS-homology 2 PTB domain; GST, glutathione-S-transferase; GAD, Gal4 activation domain; LDBD, LexA DNA binding domain; WGA, wheat germ agglutinin; PAGE, polyacrylamide gel electrophoresis; ONPG, *o*-nitrophenyl- $\beta$ -D-galactopyranoside.

incubated with glutathione-Sepharose beads (Pharmacia) for 1 h at 4 °C. Then the glutathione-Sepharose pellets were washed twice with lysis buffer and concentrated using a column. The fusion proteins were eluted with elution buffer (100 mM HEPES, pH 8, 50 mM glutathione).

**Partial Purification of Insulin Receptors**—Insulin receptors were partially purified by chromatography on wheat germ agglutinin (WGA) (26). Briefly, RHIR (Rat 1 fibroblasts expressing  $10^6$  human insulin receptors/cell) were washed twice with PBS (137 mM NaCl, 2.7 mM KCl, 6.5 mM  $\text{Na}_2\text{HPO}_4$ , 1.5 mM  $\text{KH}_2\text{PO}_4$ , pH 7.4) and solubilized for 90 min at 4 °C in 50 mM Hepes, 150 mM NaCl, 1% (v/v) Triton X-100, pH 7.6. The supernatant from an ultracentrifugation step (60 min,  $100,000 \times g$ , 4 °C) was applied to a WGA column, and insulin receptors were eluted with 0.3 M N-acetyl-D-glucosamine in 50 mM Hepes, 150 mM NaCl, 0.1% (v/v) Triton X-100, pH 7.6. Protease inhibitors were present throughout the procedure (20  $\mu\text{M}$  leupeptin, 1.25 mM bacitracin, 100 IU/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride). Insulin receptors were quantified by Scatchard analysis of  $^{125}\text{I}$ -insulin binding.

**In Vitro Phosphorylation of the Insulin Receptor Peptide 1142–1156**—This peptide has been previously characterized (27) and corresponds to the sequence 1142–1156 (TRDIYETDYRKGK) of the human insulin proreceptor (28). 300 fmol of WGA-purified insulin receptors were incubated on WGA and were activated with insulin ( $10^{-7}$  M) for 45 min. The peptide 1142–1156 was added at a final concentration of 500  $\mu\text{M}$  with a phosphorylation mixture containing 4 mM  $\text{MnCl}_2$ , 8 mM  $\text{MgCl}_2$ , 30  $\mu\text{M}$  ATP. The reaction was stopped after 1 h by spotting the total volume onto phosphocellulose papers that were dipped into 1% (v/v) orthophosphoric acid. Then the peptide was eluted in 50 mM sodium bicarbonate ( $\text{NH}_4\text{HCO}_3$ ), pH 8, for 2 h. The eluate was dried using a speed vacuum system overnight, washed twice in water, and resuspended in 200  $\mu\text{l}$  of water.

**In Vitro Phosphorylation of the IRS-2 Peptide 623–633**—This peptide contains Tyr<sup>624</sup> and Tyr<sup>628</sup> and corresponds to the sequence 623–633 (PYPEDYGDIEI) of IRS-2. Four different concentrations of peptide (50, 100, 250, and 500  $\mu\text{M}$ ) were incubated with 300 fmol of WGA-purified insulin receptors activated or not activated with insulin ( $10^{-7}$  M) and a phosphorylation mixture containing 30  $\mu\text{M}$  [ $\gamma$ - $^{32}\text{P}$ ]ATP (1.2 mCi/mmol, ICN, Irvine, CA), 4 mM  $\text{MnCl}_2$ , 8 mM  $\text{MgCl}_2$ . The phosphorylation reaction was stopped after 30 min by spotting 40  $\mu\text{l}$  onto phosphocellulose papers that were dipped into 1% (v/v) orthophosphoric acid. The papers were extensively washed, and the radioactivity incorporated into the peptide was estimated by Cerenkov counting.

**Phosphorylation of IRS-2 Fusion Proteins by the Insulin Receptor**—0.2  $\mu\text{g}$  of GST-IRS-2-KRLB, mutated or not, was incubated with 300 fmol of WGA-purified insulin receptors that were activated by insulin at  $10^{-7}$  M for 45 min in a volume of 45  $\mu\text{l}$ . To this was added 5  $\mu\text{l}$  of phosphorylation solution containing 300  $\mu\text{M}$  [ $\gamma$ - $^{32}\text{P}$ ]ATP (1.2 mCi/mmol), 40 mM  $\text{MnCl}_2$ , 80 mM  $\text{MgCl}_2$ . After 10 min, the phosphorylation reaction was stopped with Laemmli sample buffer (29). The samples were analyzed by SDS-PAGE. The gel was stained, dried, and subjected to autoradiography.

**Phosphopeptide Mapping of GST-IRS-2-KRLB, Wild Type or Mutated on both Tyr<sup>624</sup> and Tyr<sup>628</sup>**—1  $\mu\text{g}$  of GST-IRS-2-KRLB, wild type or mutated on both Tyr<sup>624</sup> and Tyr<sup>628</sup>, was incubated with 300 fmol of WGA-purified insulin receptors that were activated by  $10^{-7}$  M insulin for 20 min in 45  $\mu\text{l}$ . 5  $\mu\text{l}$  of phosphorylation mixture (300  $\mu\text{M}$  [ $\gamma$ - $^{32}\text{P}$ ]ATP (10 mCi/mmol), 40 mM  $\text{MnCl}_2$ , 80 mM  $\text{MgCl}_2$ ) was added. After 30 min, the phosphorylation reaction was stopped with Laemmli sample buffer (29). Proteins were resolved by SDS-PAGE (10%), and the gel was washed several times with  $\text{H}_2\text{O}$  to remove SDS. Then phosphorylated GST-IRS-2-KRLB, wild type and mutated, detected after 15 h of autoradiography, were excised from the gel. The gel slices were minced, washed twice with 1 ml of  $\text{H}_2\text{CO}_3\text{NH}_4$ , pH 8, and incubated overnight with the same buffer. The next day, the gel slices were subjected to tryptic digestion for a total of 24 h at 37 °C in a solution containing 1 mM  $\text{CaCl}_2$  and 50  $\mu\text{g}/\text{ml}$  trypsin. During the digestion procedure, trypsin (50  $\mu\text{g}/\text{ml}$ ) was added twice after an 8-h interval. Then the buffer containing the tryptic peptides was recovered, and peptides were washed four times with 100  $\mu\text{l}$  of  $\text{H}_2\text{O}$  and dried in a speed vacuum concentrator. Finally, the tryptic peptides were resuspended in  $\text{NH}_3$  N/1000.

The peptides were spotted onto silica thin layer chromatography plates. The first dimension was electrophoresis for 3 h at 900 V in acidic buffer containing 30% (v/v) formic acid. After drying, the plates were placed in a chromatography tank containing a buffer of 37.5% isobutyl alcohol, 25% pyridine, and 7.5% acetic acid (v/v) for 10 h. Plates were dried and subjected to autoradiography.

**Precipitation of Autophosphorylated Insulin Receptors by IRS-2 Fusion Proteins**—0.5  $\mu\text{g}$  of GST-IRS-2-KRLB, mutated or not, was incubated with 40  $\mu\text{l}$  of glutathione-Sepharose for 1 h at 4 °C. GST-IRS-2-

KRLB was used either phosphorylated or not. When phosphorylated GST-IRS-2 was used, the fusion protein-containing pellets were incubated for 30 min with insulin ( $10^{-7}$  M) and a phosphorylation mixture (30  $\mu\text{M}$  ATP, 4 mM  $\text{MnCl}_2$ , 8 mM  $\text{MgCl}_2$ ) in the presence of WGA-purified insulin receptors. Then the phosphorylated GST-IRS-2 pellets were washed five times with 0.5 M NaCl, 0.5% (v/v) Triton X-100 to remove the IR, while the unphosphorylated GST-IRS-2-KRLB pellets were washed twice with 30 mM Hepes, 30 mM NaCl, 0.1% (v/v) Triton X-100, pH 7.5. Concurrently, 2 pmol of WGA-purified insulin receptors were activated with insulin at  $10^{-7}$  M for 20 min in a final volume of 45  $\mu\text{l}$ . Thereafter, 5  $\mu\text{l}$  of phosphorylation mixture (300  $\mu\text{M}$  [ $\gamma$ - $^{32}\text{P}$ ]ATP (5 mCi/mmol), 40 mM  $\text{MnCl}_2$ , 80 mM  $\text{MgCl}_2$ ) were added. The reaction was stopped after 30 min by the addition of 500  $\mu\text{l}$  of buffer (50 mM HEPES, 150 mM NaCl, 100 mM NaF, 10 mM EDTA, and 10 mM  $\text{Na}_4\text{P}_2\text{O}_7$ ), pH 7.5. Then 500  $\mu\text{l}$  of the activated receptors were added to the pellets of phosphorylated or unphosphorylated GST-IRS-2 and incubated for 4 h. The pellets were washed three times with 30 mM Hepes, 30 mM NaCl, 0.5% (v/v) Triton X-100, pH 7.5, and the samples were analyzed by SDS-PAGE and subjected to autoradiography.

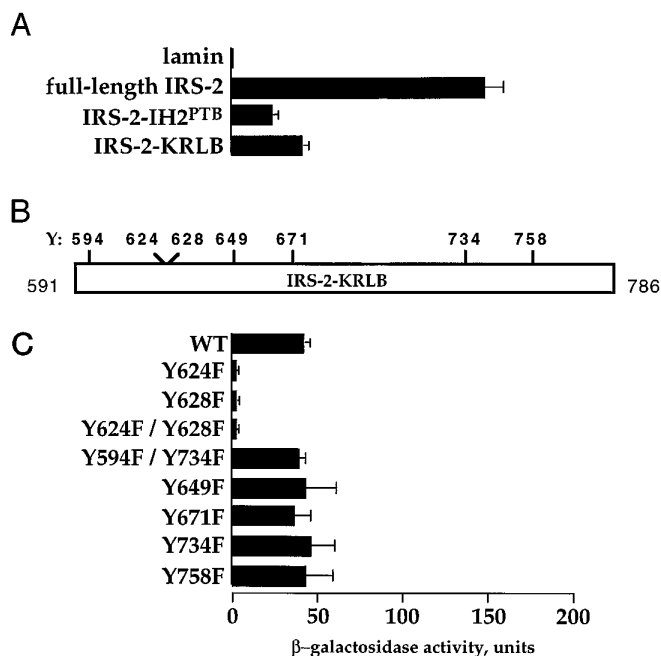
**Precipitation of Autophosphorylated IR by IRS-2 Fusion Proteins in the Presence of the IRS-2 Peptide 623–633**—Some experiments were performed in the presence of a peptide corresponding to the IRS-2 sequence 623–633. To do this, 10  $\mu\text{l}$  of IRS-2 peptide 623–633 to yield final concentrations of 10  $\mu\text{M}$ , 50  $\mu\text{M}$ , 100  $\mu\text{M}$ , 500  $\mu\text{M}$ , 750  $\mu\text{M}$ , and 1 mM were added to the IR for 1 h before mixing with the GST-IRS-2 pellets. The experiment was then performed as described above.

## RESULTS

**Tyr<sup>624</sup> and Tyr<sup>628</sup> in IRS-2 Mediate Interaction of IRS-2 with the Insulin Receptor**—In the yeast two-hybrid analysis, IRS-2 interacts with the insulin receptor through the IH2<sup>PTB</sup> and a region between residues 591 and 786 that we call the KRLB domain (13). The KRLB domain contains seven potential tyrosine phosphorylation sites at positions 594, 624, 628, 649, 671, 734, and 758. Alignment of IRS-1 and IRS-2 reveals that five of these residues are common to IRS-1, whereas Tyr<sup>628</sup> and Tyr<sup>734</sup> are unique to IRS-2 (3). To determine whether the unique tyrosine residues contribute to the interaction between the insulin receptor and the KRLB domain of IRS-2, each residue was replaced with phenylalanine, and the interaction of the mutant proteins with the cytoplasmic domain of the insulin receptor was tested in a yeast two-hybrid analysis. The KRLB domain of IRS-2 was fused to the Gal4 activation domain, whereas the catalytically active cytoplasmic portion of the insulin receptor (including the juxtamembrane region) was fused to the LexA DNA binding domain (LDBD); a IRS-2-IH2<sup>PTB</sup> fusion protein was included as a positive control. The IH2<sup>PTB</sup> and the KRLB domain interact with the insulin receptor independently during the yeast two-hybrid analysis (Fig. 1A). Furthermore, phenylalanine substitutions at Tyr<sup>624</sup> or Tyr<sup>628</sup> significantly reduced this interaction, whereas mutations at the other tyrosine residues had no effect (Fig. 1C). All of the mutant constructs interacted with a LDBD fused to N- and C-terminal Src homology 2 domains of p85 subunit of phosphatidylinositol 3-kinase, confirming that each one was expressed in the yeast cells (data not shown).

Alignment of IRS-1 and IRS-2 reveals that the KRLB domain of IRS-2 (IRS-2-(591–786)) is homologous to residues 544–751 in IRS-1; Tyr<sup>624</sup> and Tyr<sup>628</sup> in IRS-2 reside in a PY<sup>624</sup>PEDY<sup>628</sup> motif that corresponds to a SY<sup>578</sup>PEEG<sup>582</sup> motif in IRS-1 (Fig. 2). To determine the important features for insulin receptor binding, several point mutations were prepared in this motif of the IRS1-(494–741) region and the KRLB domain of IRS-2 and tested for binding to the insulin receptor in a yeast two-hybrid analysis. Substitution of Pro<sup>623</sup> in the KRLB domain with Ser<sup>623</sup> to create an IRS-1-like SYP motif significantly increased the binding to the insulin receptor (Fig. 3A); however, substitution of Ser<sup>577</sup> with proline to create an IRS-2-like PYP motif in IRS1-(494–741) did not reconstitute insulin receptor binding (Fig. 3B). Furthermore, substitution of Gly<sup>582</sup> with Tyr<sup>582</sup> in IRS1-(494–741) did not create an insulin receptor binding site

(Fig. 3B). Thus, two tyrosine residues separated by three amino acids are essential for interaction of the KRLB domain with the insulin receptor, but this binding domain cannot be reconsti-



**FIG. 1. Quantitative analysis of the interaction between the insulin receptor and IRS-2-KRLB mutants.** Measurement of the β-galactosidase activity in transformed yeast is shown. The yeast reporter strain L40 was cotransformed with a plasmid encoding the LBD-IRβ in combination with a plasmid encoding different constructs of GAD-IRS-2. Transformants were isolated on selective plates. Activation of the *LexA-LacZ* reporter gene was monitored by measuring β-galactosidase activity in cell lysates using ONPG as substrate. The activities are expressed in Miller's units (25) and are the average ± S.E. of values obtained with samples prepared from three independent transformants. **A**, cotransformations of LBD-IRβ in combination with a plasmid encoding different domains of GAD-IRS-2. As a negative control, the GAD-IRS-2-KRLB was coexpressed with the LBD-lamin hybrid. **B**, localization of tyrosine residues in IRS-2-KRLB. **C**, cotransformations of LBD-IRβ in combination with a plasmid encoding GAD-IRS-2-KRLB mutants. All IRS-2-KRLB mutants have the indicated tyrosine residues mutated to phenylalanine. WT corresponds to GAD-IRS-2-KRLB wild type.

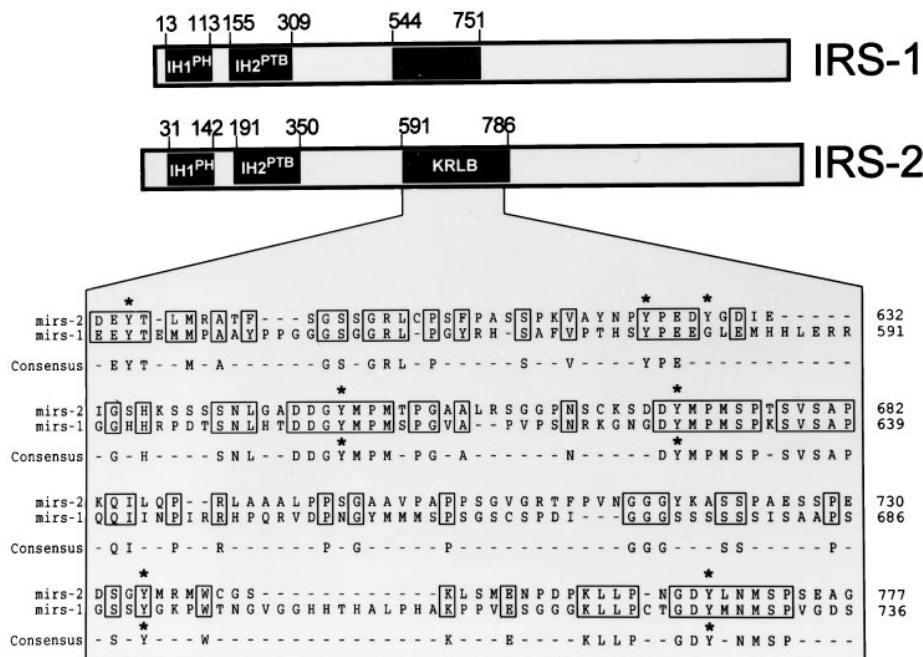
tuted in IRS-1 by various point mutations. Thus, other determinants outside of the local sequence motif are also required.

**The Role of Tyr<sup>624</sup> and Tyr<sup>628</sup> in the KRLB Domain**—The binding properties of the KRLB domain were studied *in vitro*, using GST fusion proteins containing the wild-type sequence, or mutated at position 624, position 628, or both. Unphosphorylated GST fusion proteins containing the wild-type or the mutant KRLB domain were immobilized on glutathione-Sepharose and incubated with <sup>32</sup>P-labeled WGA-purified insulin receptors. After extensive washing, the phosphorylated insulin receptor associated with the wild-type GST-KRLB domain but not with GST alone (Fig. 4). By comparison, insulin receptor binding to the GST-KRLB domain with a mutation at position 624 was reduced by 60%; mutations at Tyr<sup>628</sup> or at both Tyr<sup>624</sup> and Tyr<sup>628</sup> decreased the association to 10 and 5%, respectively.

To evaluate the contribution of Tyr<sup>624</sup> and Tyr<sup>628</sup> in the interaction of the KRLB domain with the insulin receptor, we measured the coprecipitation of the <sup>32</sup>P-labeled insulin receptor by the GST-KRLB domain in the presence of the IRS-2 peptide comprising amino acids 623–633. Within a peptide concentration range of 10–100 μM, coprecipitation of IR was reduced in a dose-dependent manner (Fig. 5). At 100 μM peptide, the amount of bound insulin receptor was reduced by 55%. A concurrent experiment carried out with a nonrelevant peptide at the same concentrations did not affect coprecipitation of IR (data not shown). Note that total inhibition of <sup>32</sup>P-labeled insulin receptor coprecipitation by GST-KRLB domain was not obtained, suggesting that additional determinants located outside of this amino acid sequence are required. This may be explained by the fact that the peptide does not adopt the same conformation as the intact domain.

**The Role of Tyr<sup>624</sup> and Tyr<sup>628</sup> in the KRLB Domain during *In Vitro* Tyrosine Phosphorylation**—To determine whether Tyr<sup>624</sup> or Tyr<sup>628</sup> is phosphorylated by the insulin receptor, the IRS-2 peptide 623–633 was used as a substrate. This peptide was not phosphorylated by the activated insulin receptor regardless of the concentrations tested (Fig. 6). However, a peptide based on the amino acid sequence of the insulin receptor kinase regulatory loop (IR 1142–1156) used commonly as an *in vitro* substrate was phosphorylated normally (Fig. 6). These results suggest that Tyr<sup>624</sup> and Tyr<sup>628</sup> in the IRS-2 peptide 623–633

**FIG. 2. Alignment of IRS-1 and IRS-2.** Sequence alignment between the KRLB domain of mouse IRS-2 and the homologous region of IRS-1 is shown. The KRLB domain contains amino acids 591–786 of IRS-2. IH1<sup>PH</sup> and IH2<sup>PTB</sup> are the pleckstrin homology domain and phosphotyrosine binding domain, respectively. Identities are boxed, and the consensus sequence is shown. The putative phosphorylation sites are labeled with asterisks.



**A**      **IRS-2: PY<sup>624</sup>PEDY<sup>628</sup>**

Coexpression of the IR with GAD fusion proteins	$\beta$ -galactosidase activity
Lamin	$1.2 \pm 0.5$
IRS-2-KRLB	$48.5 \pm 3.7$
Y624F	$1.4 \pm 0.1$
Y628F	$1.5 \pm 0.53$
P623S	$153.1 \pm 13.4$

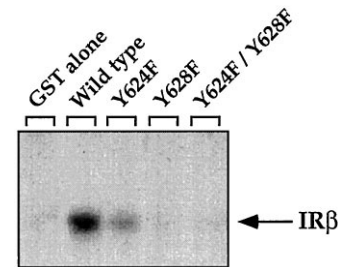
**B**      **IRS-1: SY<sup>578</sup>PEEG<sup>582</sup>**

Coexpression of the IR with GAD fusion proteins	$\beta$ -galactosidase activity
Lamin	$1.2 \pm 0.5$
IRS-1 <sup>494-741</sup>	$1.5 \pm 0.7$
S577P	$0.7 \pm 0.6$
G582Y	$1.6 \pm 0.1$

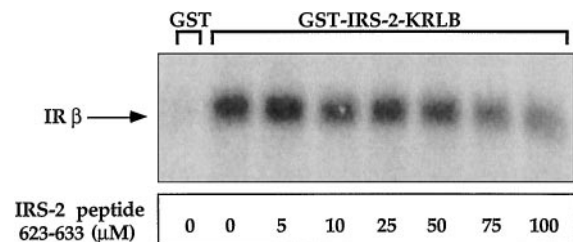
**FIG. 3. Quantitative analysis of the interaction between the insulin receptor and the PY<sup>624</sup>PEDY<sup>628</sup> motif of IRS-2-KRLB or the corresponding motif in IRS-1.** Sequence containing Tyr<sup>624</sup> and Tyr<sup>628</sup> of IRS-2 (A) and the corresponding sequence of IRS-1 (B) is shown. The yeast reporter strain L40 was cotransformed with a plasmid encoding the LBD-IR $\beta$  in combination with a plasmid encoding GAD-IRS-2-KRLB mutants (A) or GAD-IRS-1-(494–741) mutants (B). Transformants were isolated on selective plates. Activation of the *LexA-LacZ* reporter gene was monitored by measuring  $\beta$ -galactosidase activity in cell lysates using ONPG as substrate. The activities are expressed in Miller's units (25) and are the average  $\pm$  S.E. of values obtained with samples prepared from three independent transformants. IRS-2-KRLB and IRS-1-(494–741) correspond to GAD fused to the 591–786 region of IRS-2 and its corresponding region on IRS-1, respectively. All IRS mutants have the indicated amino acids mutated.

are not phosphorylated by the insulin receptor. Alternatively, it is possible that these residues are not phosphorylated in a small peptide but are phosphorylated in the intact domain, especially if conformational determinants are crucial for the interaction with the receptor.

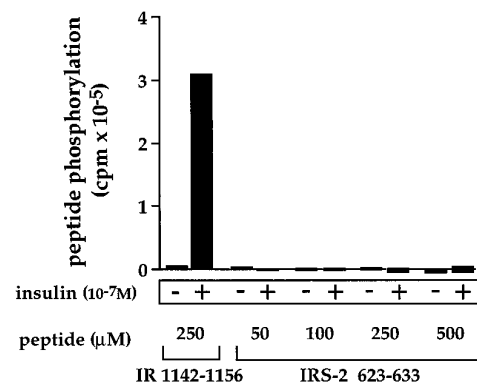
Therefore, we performed phosphopeptide maps of GST-IRS-2-KRLB, wild type or mutated on both Tyr<sup>624</sup> and Tyr<sup>628</sup>. The two fusion proteins, mutated or not, were phosphorylated by the insulin receptor in the presence of [ $\gamma$ -<sup>32</sup>P]ATP. After digestion with trypsin, they were separated by two-dimensional chromatography. A negative control experiment performed with GST alone showed that GST is not phosphorylated by the insulin receptor (data not shown). Therefore, the phosphopep-



**FIG. 4. Tyrosines 624 and 628 of IRS-2 participate in the *in vitro* binding to the IR.** Coprecipitation of the autophosphorylated IR by GST-IRS-2-KRLB wild type or mutated on Tyr<sup>624</sup> and/or Tyr<sup>628</sup> is shown. After a 20-min activation by insulin ( $10^{-7}$  M), WGA-purified IR (2 pmol/sample) was autophosphorylated for 30 min with a phosphorylation mixture containing [ $\gamma$ -<sup>32</sup>P]ATP. Then IR were added on immobilized GST-IRS-2-KRLB (0.5  $\mu$ g/sample), wild type or mutated on Tyr<sup>624</sup> and/or Tyr<sup>628</sup>. After washing, coprecipitated IR were separated on SDS-PAGE and autoradiographed. Incubation performed with GST alone is used as a negative control. A representative autoradiogram of three separate experiments with similar results is shown.



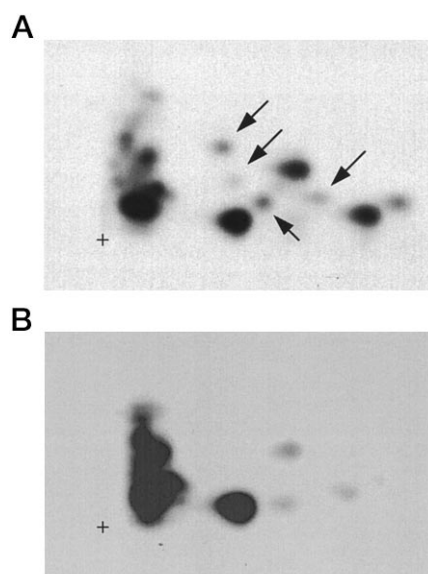
**FIG. 5. IRS-2 peptide 623–633 competes with GST-IRS-2-KRLB for binding to the IR.** <sup>32</sup>P-Labeled IR (300 fmol/sample), incubated with different concentrations (shown in  $\mu$ M) of IRS-2 peptide 623–633 were added to immobilized GST-IRS-2-KRLB (0.5  $\mu$ g/sample). IR coprecipitated with the GST fused to the KRLB domain of IRS-2 was separated by SDS-PAGE, and the gel was submitted to autoradiography. A representative autoradiogram from three separate experiments with similar results is shown.



**FIG. 6. IRS-2 peptide 623–633 is not phosphorylated by the IR.** Different concentrations of IRS-2 peptide 623–633 or IR peptide 1142–1156 were incubated with activated IR (300 fmol) and a phosphorylation mixture containing [ $\gamma$ -<sup>32</sup>P]ATP. After 30 min, the phosphorylation reaction was stopped, and the radioactivity incorporated into the peptides was counted.

tides obtained by tryptic digestion of the fusion proteins can be considered to be generated from the KRLB domain.

Analysis of autoradiograms revealed several separated phosphopeptides; a set of major peptides and a set of minor peptides localized on the two autoradiographs. This indicates that trypsin had not totally digested the proteins and that the KRLB domain of IRS-2 contains several tyrosine residues that can be phosphorylated by the insulin receptor. As shown in Fig. 7, four peptides were present only in the IRS-2-KRLB wild type autoradiogram, whereas they were absent in the Tyr<sup>624</sup> and Tyr<sup>628</sup>



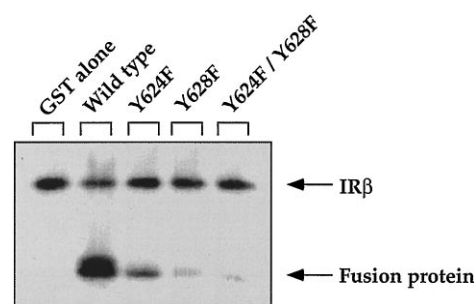
**FIG. 7. Phosphopeptide mapping by tryptic digestion of  $^{32}\text{P}$ -labeled GST-IRS-2-KRLB, wild-type or mutated on both Tyr<sup>624</sup> and Tyr<sup>628</sup>.** GST-IRS-2 KRLB, wild type or mutated on both Tyr<sup>624</sup> and Tyr<sup>628</sup>, was incubated with the IR and a phosphorylation mixture containing [ $\gamma$ - $^{32}\text{P}$ ]ATP. The phosphorylated proteins were separated on SDS-PAGE, detected by autoradiography, excised from the gel, and digested by trypsin. The peptides obtained from KRLB wild type domain (A) or KRLB double mutant domain (B) were spotted onto silica plates, which were subjected to electrophoresis and then to thin layer chromatography. Plates were dried and submitted to autoradiography. The origin of migration is indicated by a *plus sign*, and the additional phosphopeptides are identified by arrows.

mutated protein. This suggests that Tyr<sup>624</sup> and/or Tyr<sup>628</sup> in the KRLB domain of IRS-2 may be phosphorylated by the insulin receptor.

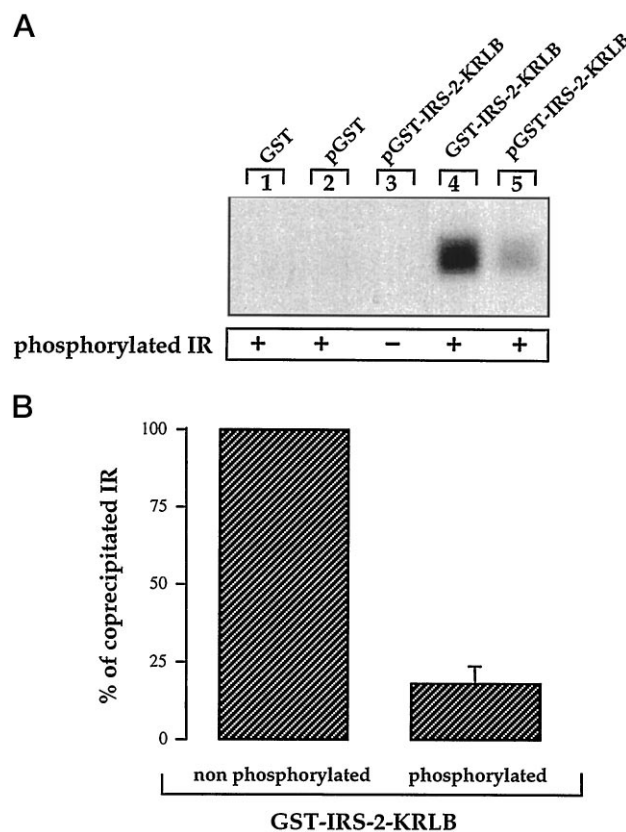
To determine the role of Tyr<sup>624</sup> and Tyr<sup>628</sup> for phosphorylation of the KRLB domain, the wild-type or mutated GST fusion proteins were incubated with [ $\gamma$ - $^{32}\text{P}$ ]ATP and WGA-purified insulin receptors. The proteins were separated by SDS-PAGE, and the phosphorylation of the insulin receptor  $\beta$ -subunit and the GST-KRLB domain fusion proteins was monitored by autoradiography. The wild-type KRLB domain was phosphorylated by the insulin receptor, whereas its phosphorylation was reduced to 50, 20, and 10% by mutations at Tyr<sup>624</sup>, Tyr<sup>628</sup>, and both positions, respectively (Fig. 8). Thus, Tyr<sup>624</sup> and Tyr<sup>628</sup> are involved in the interaction between the IR and the KRLB domain of IRS-2, including tyrosine phosphorylation, and Tyr<sup>628</sup> seems to be more important than Tyr<sup>624</sup> in this process.

**The Interaction between the IR and KRLB Domain Decreases When IRS-2 Is Phosphorylated**—Next we examined whether phosphorylation of the KRLB domain modifies the *in vitro* binding to the insulin receptor. Immobilized GST-KRLB domain was phosphorylated or not phosphorylated by activated WGA-purified IR and then extensively washed to remove the receptor; no  $^{32}\text{P}$ -labeled insulin receptor was retained after stringent washes (Fig. 9A, lane 3). Thereafter, the immobilized KRLB domain was incubated with fresh samples of WGA-purified insulin receptor autophosphorylated in the presence of [ $\gamma$ - $^{32}\text{P}$ ]ATP in separate incubations. GST alone did not coprecipitate the  $^{32}\text{P}$ -labeled insulin receptor (lanes 1 and 2). However, the unphosphorylated GST-KRLB domain bound strongly to the  $^{32}\text{P}$ -labeled insulin receptor (lane 4). In contrast, the binding of the phosphorylated GST-KRLB domain to the insulin receptor was reduced by 80% ( $p < 5 \times 10^{-4}$ ) (lane 5 and Fig. 9B).

To ascertain that this result is not due to contamination of the WGA preparation by proteases and/or by kinases different



**FIG. 8. Tyrosines 624 and 628 of IRS-2 participate in the *in vitro* tyrosine phosphorylation of IRS-2-KRLB.** GST-IRS-2-KRLB (0.2  $\mu\text{g}/\text{sample}$ ) wild type or mutated on Tyr<sup>624</sup> and/or Tyr<sup>628</sup> were incubated with WGA-purified IR (300 fmol/sample) activated with insulin ( $10^{-7}$  M) in the presence of phosphorylation mixture containing [ $\gamma$ - $^{32}\text{P}$ ]ATP. After 10 min, the reaction was stopped with 50  $\mu\text{l}$  of Laemmli sample buffer. The samples were analyzed by SDS-PAGE and autoradiographed. A representative autoradiogram of four separate experiments with similar results is shown.



**FIG. 9. Phosphorylation of IRS-2-KRLB decreases the interaction with the IR.** GST alone (lanes 1 and 2) or GST-IRS-2-KRLB (lanes 3, 4, and 5) (0.5  $\mu\text{g}/\text{sample}$ ) immobilized on glutathione-Sepharose were phosphorylated (lanes 2, 3, and 5) or not (lanes 1 and 4) by activated IR. The GST pellet, extensively washed to remove the receptor, was incubated with  $\gamma$ - $^{32}\text{P}$ -labeled IR (300 fmol/sample, lane 3). Coprecipitated IR were separated by SDS-PAGE and autoradiographed. A, a representative autoradiogram from three independent experiments with similar results. B, results are expressed as a percentage of coprecipitated IR by nonphosphorylated GST-IRS-2-KRLB and are the mean  $\pm$  S.E. of three different experiments.

from the insulin receptor, we performed experiments using immunopurified insulin receptors. We obtained similar results, indicating that the difference between the phosphorylated and the nonphosphorylated forms of GST-KRLB is likely to be due to insulin receptor-induced phosphorylation (data not shown).

**The Regulatory Loop of the Insulin Receptor Interacts with the KRLB Domain**—We have previously shown (13) that the

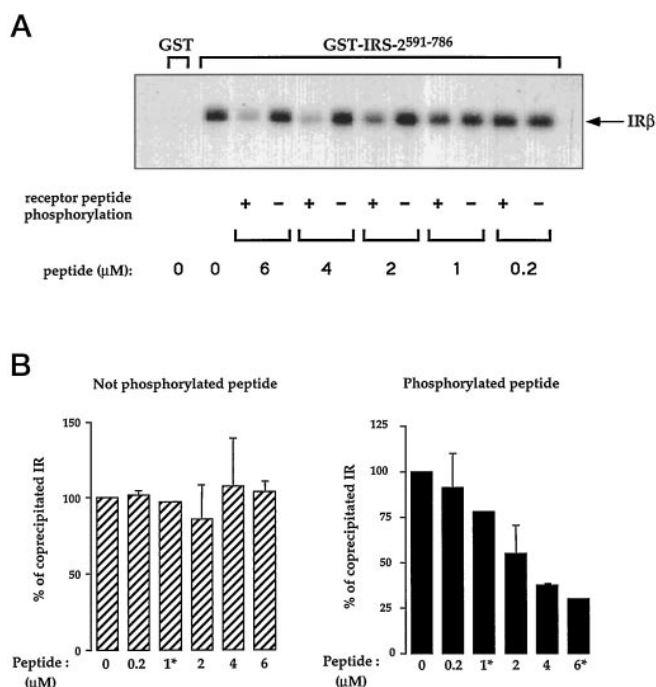


FIG. 10. Competition between the IR and a phosphorylated or nonphosphorylated IR peptide containing Tyr<sup>1146</sup>, Tyr<sup>1150</sup>, and Tyr<sup>1151</sup>. The GST-IRS-2-(591-786) containing the KRLB domain of IRS-2 (0.5 μg/sample) was incubated with <sup>32</sup>P-labeled IR (300 fmol/sample) in the absence or in the presence of a peptide containing amino acids 1142-1156. The phosphorylated peptide or the nonphosphorylated one was added at different concentrations (shown in μM). IR coprecipitated with the GST fused to IRS-2-KRLB was separated by SDS-PAGE and autoradiographed (A). IR-associated <sup>32</sup>P radioactivity was quantified (B). Results are expressed as a percentage of IR coprecipitated in the absence of peptide and are the mean ± S.E. of three different experiments. \*, a value of two different experiments.

autophosphorylation sites in the regulatory loop of the IR kinase (Tyr<sup>1146</sup>, Tyr<sup>1150</sup>, and Tyr<sup>1151</sup>) are required for the interaction between the IR and KRLB domain. To address this issue in more depth, we measured the coprecipitation of the IR by the GST-KRLB domain in the presence of a peptide containing Tyr<sup>1146</sup>, Tyr<sup>1150</sup>, and Tyr<sup>1151</sup>. Different concentrations of peptide corresponding to the sequence 1142-1156, phosphorylated or not, were incubated with the GST-KRLB domain immobilized on glutathione-Sepharose. At all concentrations tested, the nonphosphorylated peptide had no effect on receptor binding to the GST-KRLB domain (Fig. 10). In contrast, when the peptide was phosphorylated, competition between the insulin receptor and the peptide was observed, and the amount of insulin receptor bound to the GST-KRLB domain was reduced in a dose-dependent manner (Fig. 10B). These results suggest that the KRLB domain binds to the phosphorylated regulatory loop of the insulin receptor.

#### DISCUSSION

In addition to the IH2<sup>PTB</sup> in IRS-1 and IRS-2 that engages the phosphorylated NPEY motif in the insulin receptor juxtamembrane region, we identified a second region in IRS-2 between residues 591 and 786 that binds to the insulin receptor (13). Recently, He *et al.* (16) reported similar results. Since this novel region interacts with the regulatory loop of the insulin receptor, we call it the kinase regulatory loop binding, or KRLB, domain. The KRLB domain in IRS-2 provides a unique mechanism of interaction between IRS-2 and the insulin receptor, which is absent from IRS-1. Insulin-stimulated phosphorylation of the regulatory loop plays an important role in activation of the insulin receptor kinase (30). Mutations in this

region decrease the kinase activity of the receptor *in vitro* and *in vivo* and reduce the strength of the insulin biological responses (31, 32). The finding that the regulatory loop may also play an important role for IRS-2 binding functionally couples the activation step to substrate selection. The absence of the KRLB domain from IRS-1 may facilitate the formation of the flexible complex, which is more easily phosphorylated by the insulin receptor. In contrast, by engaging IRS-2 through both the IH2<sup>PTB</sup> domain and the KRLB domain, the degrees of freedom in the complex may be reduced and restrict access to certain tyrosine phosphorylation sites. This difference between IRS-1 and IRS-2 is likely to generate differences in the tyrosine phosphorylation pattern of these substrates. Hence, this mechanism could result in different signaling potentials for IRS-1 and IRS-2.

Our experiments suggest that Tyr<sup>624</sup> and Tyr<sup>628</sup> in the KRLB domain are essential for binding to the insulin receptor. Substitution of these residues individually or together with phenylalanine inhibited insulin receptor binding, whereas mutations at the other tyrosine residues in this domain did not affect the interaction. Since the unphosphorylated peptide inhibits the interaction between the KRLB domain and the insulin receptor, we conclude that Tyr<sup>624</sup> and Tyr<sup>628</sup> function in an unphosphorylated state to promote the interaction.

The yeast two-hybrid analysis reveals that the interaction between the KRLB domain and the insulin receptor requires a functional kinase domain and all three phosphorylation sites (Tyr<sup>1146</sup>, Tyr<sup>1150</sup>, and Tyr<sup>1151</sup>) in the regulatory loop of the insulin receptor (13). In this case, a phosphopeptide corresponding to the amino acid sequence in the regulatory loop inhibits the binding of the KRLB domain to the insulin receptor, whereas the unphosphorylated peptide does not interfere. Together with the yeast two-hybrid analysis, these results suggest that the Tris-phosphorylated regulatory loop constitutes an interaction site for the KRLB domain of IRS-2. Although the peptide competition experiments favor this hypothesis, it is possible that the phosphorylation of the regulatory loop exposes another region that is masked in the unstimulated receptor.

From our results, we propose the following model. The IRS-2<sup>PTB</sup> domain would be the primary anchor of IRS-2 to the insulin receptor; the KRLB domain of IRS-2, including the PY<sup>624</sup>PEDY<sup>628</sup> motif, would stabilize this binding to the insulin receptor, which then phosphorylates tyrosines of this domain. When we compared the amount of insulin receptor coprecipitated by the GST-KRLB phosphorylated or not phosphorylated, we found that phosphorylation of IRS-2-KRLB decreased the interaction with the insulin receptor. Hence, it would appear that the binding between the insulin receptor and the KRLB domain of IRS-2 results in tyrosine phosphorylation of the KRLB domain, and this leads to decreased binding of IRS-2 to the insulin receptor. It is tempting to think that such a mechanism might be involved in releasing IRS-2 from the receptor.

Although IRS-1 contains significant amino acid sequence homology to the KRLB domain of IRS-2, it does not bind to the regulatory loop of the insulin receptor (13). The PY<sup>624</sup>PEDY<sup>628</sup> motif in IRS-2 that contains these residues is similar but not completely conserved in IRS-1 (SY<sup>578</sup>PEEG<sup>582</sup>). Comparison of the two sequences shows that they contain three different residues, two of which (proline 623 and tyrosine 628) are differently charged. However, mutations converting this motif in IRS-1 to resemble the motif in IRS-2 did not create a KRLB domain in IRS-1. In particular, substitutions of Gly<sup>582</sup> with Tyr did not create the binding site, although *in vitro* binding experiments suggest that Tyr<sup>628</sup> is the predominant functional residue in the KRLB domain. Moreover, mutation of Pro<sup>623</sup> to

Ser<sup>623</sup> in IRS-2 enhances rather than diminishes the binding of the KRLB domain to the insulin receptor. This result indicates that the particular structure induced by the prolines surrounding Tyr<sup>624</sup> is not a major determinant for the IRS-2 interaction with the insulin receptor. This suggests that the Tyr<sup>624</sup> and Tyr<sup>628</sup> in the KRLB domain mediate the insulin receptor binding, but other regions of the KRLB domain that are absent from IRS-1 must create the binding pocket.

In conclusion, in addition to the pleckstrin homology domain and the phosphotyrosine binding domain in IRS-1 and IRS-2, IRS-2 has the region called the KRLB domain comprising amino acids 591–786 for interacting with the insulin receptor. Knowing that this region is absent in IRS-1 and that the IRS-molecules are substrates for the kinases associated to receptors for cytokines and GH (33–40), it is tempting to think that these differences between IRS-1 and IRS-2 might contribute to a unique regulation by the different receptor and nonreceptor tyrosine kinases.

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