Phosphorylation of Ser$^{307}$ in Insulin Receptor Substrate-1 Blocks Interactions with the Insulin Receptor and Inhibits Insulin Action*

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Serine phosphorylation of insulin receptor substrate-1 (IRS-1) inhibits insulin signal transduction in a variety of cell backgrounds, which might contribute to peripheral insulin resistance. However, because of the large number of potential phosphorylation sites, the mechanism of inhibition has been difficult to determine. One serine residue located near the phosphotyrosine-binding (PTB) domain in IRS-1 (Ser$^{307}$ in rat IRS-1 or Ser$^{312}$ in human IRS-1) is phosphorylated via several mechanisms, including insulin-stimulated kinases or stress-activated kinases like JNK1. During a yeast tri-hybrid assay, phosphorylation of Ser$^{307}$ by JNK1 disrupted the interaction between the catalytic domain of the insulin receptor and the PTB domain of IRS-1. In 32D myeloid progenitor cells, phosphorylation of Ser$^{307}$ inhibited insulin stimulation of the phosphatidylinositol 3-kinase and MAPK cascades. These results suggest that inhibition of PTB domain function in IRS-1 by phosphorylation of Ser$^{307}$ (Ser$^{312}$ in human IRS-1) might be a general mechanism to regulate insulin signaling.

The insulin signaling system plays an important role in many physiological processes, including carbohydrate and fat metabolism, reproduction, cellular growth, and survival (1). Acute insulin resistance is mediated, at least in part, by the action of pro-inflammatory cytokines that are produced during infection, physical trauma, or cancer (2–4). Chronic insulin resistance is an inevitable consequence of genetic variation that is exacerbated by aging and obesity and contributes to multiple disorders, including glucose intolerance, hyperlipidemia, hypertension and cardiovascular mortality, infertility and polycystic ovarian syndrome, and type II diabetes (5, 6). Insulin resistance alone might not cause diabetes if pancreatic β-cells secrete enough insulin to compensate for reduced sensitivity; however, type II diabetes eventually develops, possibly because hyperinsulinemia itself exacerbates the pre-existing resistance until β-cells eventually fail to compensate (7). Understanding the molecular basis of insulin resistance will provide a rational basis for treatment of many related disorders.

The insulin signaling system is complex, and a common mechanism to explain the occurrence of acute and chronic insulin resistance is difficult to identify. Mutations in the insulin receptor are an obvious source of lifelong insulin resistance, but they occur rarely and are not the common cause of type II diabetes (8–11). Generally, insulin resistance is a consequence of dysregulated insulin signaling that arises from various sources. Nonspecific or regulated degradation of elements in the insulin signaling pathway might cause insulin resistance (12); elevated activity or expression of protein or lipid phosphatases, including PTP1B, SHIP2, and pTen, directly inhibits insulin signals (13, 14). Covalent modification of the IRS1 proteins by serine phosphorylation is implicated in insulin resistance associated with obesity and trauma. Serine phosphorylation of IRS-1 is known to be promoted by elevated circulating levels of several metabolites, including free fatty acids, diacylglycerol, fatty acyl-CoAs, ceramides, and glucose (15). Moreover, adipose-derived cytokines like TNF-α also stimulate serine/threonine phosphorylation of IRS-1, which inhibits signaling (16).

One of the branches of the TNF-α signaling pathway involves activation of JNK (17–19). JNK phosphorylates numerous cellular proteins, including IRS-1, IRS-2, Shc, and Gab-1 (20). Previous work has revealed that the major JNK phosphorylation site in rat IRS-1 is located at Ser$^{307}$ (Ser$^{312}$ in human IRS-1), which is located on the C-terminal side of the phosphotyrosine-binding (PTB) domain (20). In this report, a yeast tri-hybrid assay revealed that JNK1 phosphorylation of Ser$^{307}$ inhibits the interaction between IRS-1 and the insulin receptor, providing a rational mechanism to explain, at least in part, the insulin resistance that occurs during trauma and obesity.

MATERIALS AND METHODS

Antibodies and Reagents—Phospho-specific MAPK, control MAPK, and phospho-specific Akt antibodies were purchased from New England Biolabs Inc. Control anti-Akt and anti-JNK1 antibodies were purchased from Santa Cruz Biotechnology. Antibodies against IRS-1, IRS-2, and p85 have been described (21, 22). Antibodies directed against phosphorylated Ser$^{312}$ in IRS-1 were purchased from BIO-SOURCE. Rabbit polyclonal serum directed against phosphorylated Ser$^{307}$ was generated using a synthetic peptide designed to contain phosphorylated Ser$^{307}$ and surrounding amino acids (Boston Biomolecules). Insulin was purchased from Roche Molecular Biochemicals. IGF-1 was a gift from Lilly. TNF was purchased from R&D Systems. IRS-1 tyrosine phosphorylation site mutants have been previously described (23, 24). Point mutants for Ser$^{307}$ and in the JNK-binding domain of IRS-1 were generated using appropriate oligonucleotides.

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1 The abbreviations used are: IRS, insulin receptor substrate; TNF, tumor necrosis factor; JNK, c-Jun N-terminal kinase; PTB, phosphotyrosine-binding; MAPK, mitogen-activated protein kinase; IGF-1, insulin-like growth factor-1; GST, glutathione S-transferase; JIP, JNK-interacting protein; PI3K, phosphatidylinositol 3-kinase; IR, insulin receptor; SD, synthetic dextrose; ERK, extracellular signal-regulated kinase; PH, pleckstrin homology; MEK, MAPK/ERK kinase.

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with the Stratagene QuikChange site-directed mutagenesis method. JNK1 and GST-JIP JNK-binding domain constructs have been described (25, 26).

**Cell Culture**—32D cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, 5% WEHI conditioned medium (as a source of interleukin-3), and 5 mM histidinol and made quiescent by serum starvation for 4 h. 32D transfecteds were generated by electroporation and selected in histidinol as previously described (27). HEK293 cells were maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum and made quiescent by serum starvation for 12 h.

**Cell Lysis, Immunoprecipitation, and Western Analysis**—32D cells were lysed in 50 mM Tris (pH 7.4) containing 130 mM NaCl, 5 mM EDTA, 1% Nonidet P-40, 100 mM NaF, 50 mM β-glycerophosphate, 100 μM NaVO₄, 1 mM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, and 5 μg/ml aprotinin. Immunoprecipitations were performed for 2 h at 4 °C, followed by collection on protein A-Sepharose. Lysates and immunoprecipitates were resolved by SDS-PAGE and transferred to nitrocellulose, and proteins were detected by immunoblotting and either 125I-labeled protein A or enhanced chemiluminescence (Amersham-Pharmacia) and analysis by autoradiography or on a Molecular Dynamics PhosphorImager. HEK293 cells were lysed in 20 mM Tris (pH 7.4) containing 137 mM NaCl, 25 mM β-glycerophosphate, 2 mM sodium pyrophosphate, 2 mM EDTA, 1% Triton X-100, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, 5 μg/ml aprotinin, 2 mM benzamidine, and 0.5 mM dithiothreitol.

**Association of IRS-1 with JNK1**—GST fusion proteins containing portions of IRS-1 were made by subcloning the indicated residues into pGEX-2TK (Amersham Biosciences, Inc.), expressed in Escherichia coli (BL21), and purified using glutathione-agarose (Amersham Biosciences, Inc.). GST fusion proteins (111 pmol) were incubated with 293 cell lysates for 2 h at 4 °C. Proteins bound to the GST fusion proteins were fractionated by SDS-PAGE, transferred to nitrocellulose, and analyzed by Western blotting with antibodies against JNK1.

**In Vitro Kinase Assay**—HEK293 cells were transiently transfected with either pcDNA3-FLAG-JNK1 or pcDNA3 using Fugene-6. Transient transfecteds were made quiescent by serum starvation for 12 h and assayed at 36 h. Following stimulation with 10 μg/ml anisomycin and lysis, FLAG-JNK1 was immunoprecipitated with M2 antibody (Sigma) for 2 h at 4 °C, and immune complexes were collected on anti-mouse agarose (Sigma). FLAG-JNK1 was eluted with FLAG peptide (100 μg/ml) in kinase buffer (25 mM Hepes (pH 7.4), 25 mM β-glycerophosphate, 25 mM MgCl₂, 100 μM sodium orthovanadate, and 0.5 mM dithiothreitol) overnight at 4 °C. Kinase assays were initiated by addition of kinase and 50 μM [γ-32P]ATP to baculovirus-expressed insulin receptor in a final volume of 50 μl of kinase buffer. Reactions were terminated after 30 min at 22 °C with ice-cold phosphate-buffered saline and addition of SDS sample buffer. After SDS-PAGE and transfer to nitrocellulose, 32P phosphorylation of substrate proteins was examined by autoradiography and Cerenkov counting. PI3K activity assays were performed on IRS-1 immunoprecipitates as previously described (28).

**RESULTS**

Phosphorylation of Ser³⁰⁷ in IRS-1—IRS-1 of rat or human origin contains many potential serine phosphorylation sites that are thought to play regulatory roles during insulin signaling. One of these sites, Ser³⁰⁷ in rat IRS-1, was originally found to be phosphorylated specifically by JNK. Ser³⁰⁷ was later found to be phosphorylated in IRS-1 isolated from cells and tissues stimulated with TNF-α, insulin/IGF-1, or anisomycin (20, 29). Phosphorylation of Ser³⁰⁷ is interesting because it inhibits insulin-stimulated tyrosine phosphorylation of rat IRS-1. To study the role of Ser³⁰⁷ in insulin signaling, full-length rat IRS-1 or a mutant IRS-1 molecule containing a Ser³⁰⁷ → Ala substitution (A307IRS-1) was stably expressed in 32D cells. 32D cells are interleukin-3-dependent murine myeloid progenitor cells that express few insulin receptors and no IRS proteins; overexpression of IRS-1 and the insulin receptor reconstitutes many aspects of the insulin signaling pathway in 32D cells (26, 30).

Immunoprecipitates of IRS-1 from 32D cells stimulated with insulin or anisomycin were analyzed by immunoblotting with phospho-specific antibodies against Ser³⁰⁷ (αpS³⁰⁷) and, for comparison, Ser³⁰⁸ (αpS³⁰⁸). Before stimulation, both antibodies reacted weakly with IRS-1, indicating that these phosphorylation sites were slightly phosphorylated under the basal conditions (Fig. 1A). Anisomycin or insulin strongly stimulated phosphorylation of Ser³⁰⁷ and Ser³⁰⁸, whereas Ser³⁰⁸ was phosphorylated only during anisomycin stimulation (Fig. 1A). As previously shown (20), TNF-α stimulated Ser³⁰⁷ phosphorylation more slowly than either insulin or anisomycin (Fig. 1B).
Phosphorylation of Rat IRS-1

Phosphorylation of Ser307 in 32DIR cells (Fig. 2). To determine whether insulin-stimulated Ser307 phosphorylation was restored by site-directed mutagenesis of IRS-1 during insulin stimulation, since Ser307 is near the PTB domain of IRS-1, phosphorylation of this residue during insulin or TNF-α stimulation might disrupt the interaction between the insulin receptor and IRS-1. To determine whether JNK1-mediated phosphorylation of Ser307 inhibits binding between the insulin receptor and IRS-1, a yeast tri-hybrid assay was developed to test the effect of JNK1 on the interaction between the insulin receptor (bait) and various IRS-1 constructs (prey).

Prior work revealed that the PTB domain couples the IR to IRS-1 in yeast (33); however, to validate the tri-hybrid assay, the specific interaction between JNK1 and IRS-1 in yeast was established. Human IRS-1 contains two putative JIP homology motifs between residues 785 and 791 and residues 857 and 863 (residues 780 and 786 and residues 852 and 858 in the rat ortholog) that might specifically bind JNK1 (Fig. 3A) (20, 36). The LXXLL sequence of this motif in JIP-1 is required for JNK recruitment and activation (26). To establish which motif interacts with JNK1, various deletion constructs of IRS-1 (prey) were expressed with JNK1 (bait) in the yeast two-hybrid assay. Full-length IRS-1 interacted with JNK1 as revealed by β-galactosidase activity in yeast growing on selective medium, whereas an IRS-1 structure lacking both JIP homology regions did not promote growth and β-galactosidase activity (Fig. 3B). These results suggest that the JIP homology motif in human IRS-1 between residues 857 and 863 binds to JNK1 in the yeast two-hybrid system.

In vitro binding experiments confirmed that the orthologous JIP homology region (RPTTRSL, motif) in rat IRS-1 binds JNK1. GST fusion proteins containing a portion of rat IRS-1 with intact or mutant JIP homology domains were incubated with 293 cell lysates containing JNK1. JNK1 associated with the immobilized fragments of wild-type IRS-1 (Fig. 3C). How-
ever, a Leu\textsuperscript{852/858} → Gly substitution, but not a Leu\textsuperscript{784/786} → Gly substitution, abolished the ability of rat IRS-1 to pull-down JNK1 (Fig. 3C).

Yeast cells expressing the human insulin receptor (bait) and human IRS-1 constructs (prey) grew efficiently on selective medium, and growth was not inhibited when inactive JNK1 (JNK1\textsuperscript{APF}) or an empty vector control (EV) was expressed in these yeast cells (Fig. 3D). By contrast, coexpression of active JNK1 prevented growth, suggesting that the IR/IRS-1 interaction was inhibited (Fig. 3D). Substitution of Ser\textsuperscript{312} for alanine in human IRS-1 (orthologous to Ser\textsuperscript{307} in rat IRS-1) blocked JNK1-mediated growth inhibition, suggesting that JNK1-mediated phosphorylation of Ser\textsuperscript{307} is required for the JNK1-mediated disruption of the IR/IRS-1 interaction. Moreover, truncated human IRS-1 composed of residues 45–516, including Ser\textsuperscript{312} but lacking the JIP homology region, was insensitive to JNK1-mediated disruption of the IR/IRS-1 interaction (Fig. 3D). The insulin receptor did not interact with JNK1 in a yeast two-hybrid assay (data not shown) and was not phosphorylated by JNK1 during \textit{in vitro} kinase assays using purified insulin receptor and JNK1 (Fig. 3E). These results are consistent with the hypothesis that the interaction between the insulin receptor and IRS-1 is inhibited by phosphorylation of Ser\textsuperscript{307} during association of JNK1 with the JIP homology region of IRS-1.

Phosphorylation of Ser\textsuperscript{307} Inhibits Insulin Signaling—Anisomycin was used to promote phosphorylation of Ser\textsuperscript{307} in rat IRS-1 and A307\textsuperscript{IRS-1} to establish the effect on insulin-stimulated PI3K and MAPK cascades. Anisomycin rapidly stimulated Ser\textsuperscript{307} phosphorylation of IRS-1, with a half-maximal effect below 100 ng/ml (Fig. 4, A and B). By contrast, \textit{opS}\textsuperscript{307}, weakly immunoblotted A307\textsuperscript{IRS-1} before and after anisomycin treatment, confirming that Ser\textsuperscript{307} was removed from the mutant molecule (Fig. 4, A and B). The residual immunoblotting of A307\textsuperscript{IRS-1} by \textit{opS}\textsuperscript{307} might reflect cross-reactivity with other phosphorylation sites in IRS-1, such as Ser\textsuperscript{612}.

Tyrosine phosphorylation of IRS-1 was detected by immunoblotting with anti-phosphotyrosine antibodies as previously described (37). Treatment of 32D\textsuperscript{IR/IRS-1} cells for 30 min with...
0.01 or 0.1 μg/ml anisomycin inhibited insulin-stimulated tyrosine phosphorylation by 35%, and inhibition reached 45% with 1.0 μg/ml anisomycin (Fig. 5A). Low concentrations of anisomycin had no inhibitory effect on tyrosine phosphorylation of A307IRS-1, and inhibition barely reached 15% at 1.0 μg/ml anisomycin (Fig. 5A). The inhibitory effect of anisomycin did not occur through degradation of IRS-1 or inhibition of insulin receptor autophosphorylation (data not shown). However, anisomycin-mediated inhibition of IRS-1 tyrosine phosphorylation required a functional JIP homology domain. Mutation of the LXL sequence of the JNK-binding domain of JIP-1 to GXG abrogates JNK interaction (26). Inactivation of the JIP homology region by point mutations of the L856SL motif to a G856SG motif completely eliminated the inhibitory effect of anisomycin (Fig. 6). These results confirm that the intact JIP homology region mediates inhibition of insulin-stimulated tyrosine phosphorylation, most likely through phosphorylation of Ser307.

Many insulin signals are mediated through the binding of tyrosine-phosphorylated motifs in IRS-1 to the SH2 domains in various signaling proteins (SH2 proteins), including PI3K and Grb2 (38). Consistent with the inhibition of insulin-stimulated tyrosine phosphorylation of IRS-1, anisomycin inhibited the binding of p85 to IRS-1 in 32D18 cells; however, the binding of A307IRS-1 to p85 during insulin stimulation was not inhibited by anisomycin (Fig. 5B). In the same dose-dependent manner, anisomycin inhibited insulin-stimulated PI3K activity associated with IRS-1, but had no effect on PI3K activity associated with A307IRS-1 during insulin stimulation (Fig. 5C).

Insulin promotes the association of Grb2 with IRS-1 or Shc, which stimulates the phosphorylation of ERK1 and ERK2, as detected by immunoblotting with anti-phospho-MAPK antibodies (1). In 32D18 cells, analysis of the inhibitory effect of anisomycin on ERK1 phosphorylation was confounded by stimulation of ERK1 phosphorylation by anisomycin and insulin.

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**FIG. 4.** Anisomycin induces phosphorylation of Ser307 in IRS-1 in a dose- and time-dependent manner. A, IRS-1 immunoprecipitates from 32DIRD, 32DIRD/IRS-1, and 32DIRD/A307IRS-1 cells treated with 1.0 μg/ml anisomycin for the indicated times were analyzed by immunoblotting with pS307 and anti-IRS-1 antibody. B, IRS-1 immunoprecipitates from 32DIRD, 32DIRD/IRS-1, and 32DIRD/A307IRS-1 cells treated with the indicated doses of anisomycin (Aniso) for 30 min were analyzed by immunoblotting with pS307 and anti-IRS-1 antibody.

**FIG. 5.** Ser307 in IRS-1 is required for inhibition of insulin signaling by anisomycin. A, proteins in whole cell lysates from 32DIRD/IRS-1 and 32DIRD/A307IRS-1 cells treated with the indicated doses of anisomycin (Aniso) for 30 min prior to stimulation with 10 nM insulin for 5 min were analyzed with anti-phosphotyrosine antibodies (aPY). B, immunoprecipitates (IP) of the p85 regulatory subunit of PI3K from 32DIRD/IRS-1 and 32DIRD/A307IRS-1 cells treated with the indicated doses of anisomycin for 30 min prior to stimulation with 10 nM insulin for 5 min were analyzed with anti-IRS-1 antibody. C, IRS-1 immunoprecipitates from 32DIRD/IRS-1 and 32DIR/A307IRS-1 cells treated with the indicated doses of anisomycin for 30 min prior to stimulation with 10 nM insulin for 5 min were analyzed for associated PI3K activity. Phosphorylated inositol was resolved by chromatography and visualized by phosphorimaging.
IRS-1 Ser\textsuperscript{307} Phosphorylation Blocks IR/IRS-1 Interaction

through the Shc pathway in the absence of IRS-1 or A307\textsuperscript{IRS-1} expression (Fig. 7A). By contrast, ERK2 was phosphorylated only during insulin stimulation of 32D\textsuperscript{IR} cells expressing either IRS-1 or A307\textsuperscript{IRS-1} (Fig. 7, A and B). Anisomycin completely inhibited insulin stimulation of ERK2 phosphorylation (60% inhibition at 0.1 \textmu g/ml) in 32D\textsuperscript{IR}/IRS-1 cells. By contrast, ERK2 phosphorylation was barely inhibited (20%) at the highest anisomycin concentration in 32D/A307\textsuperscript{IRS-1} cells (Fig. 7B). These data reveal that phosphorylation of Ser\textsuperscript{307} inhibits IRS-1-mediated ERK2 phosphorylation.

**DISCUSSION**

Our results reveal a general mechanism for the negative feedback and heterologous regulation of the IRS-1 branch of the insulin signaling pathway through inhibition of PTB domain function by phosphorylation of Ser\textsuperscript{307}. Previous work established that the interaction in yeast between the insulin receptor catalytic domain and IRS-1 is mediated entirely through the binding of the phosphorylated NPEY motif in the insulin receptor to the PTB domain in IRS-1 (32, 33, 39). Based on this prior information, we conclude that disruption of the binding between the insulin receptor and IRS-1 in yeast expressing JNK1 occurs because phosphorylation of Ser\textsuperscript{307} disrupts PTB domain function (34, 35). All of the control experiments confirmed this conclusion, including association of the insulin receptor and IRS-1 in yeast expressing a kinase-dead JNK1 construct, association of the insulin receptor and a human IRS-1 mutant (Ser\textsuperscript{312} \rightarrow Ala) in yeast expressing a functional JNK1 construct, and association of the insulin receptor and an IRS-1 construct lacking the JIP homology region in yeast expressing JNK1.

Although the yeast tri-hybrid assay reveals that Ser\textsuperscript{307} phosphorylation completely abrogates insulin receptor/IRS-1 interaction, the 32D\textsuperscript{IR} cell-based experiments suggest that it inhibits IRS-1 tyrosine phosphorylation by only 50% at best.

**FIG. 6.** Proteins in whole cell lysates from 32D\textsuperscript{IR}/IRS-1 and 32D\textsuperscript{IR}/GSGIRS-1 cells treated with the indicated doses of anisomycin (Aniso) for 30 min prior to stimulation with 10 nM insulin for 5 min were analyzed with antibodies against phospho-MAPK (pMAPK). IP, immunoprecipitate GSG\textsuperscript{IRS-1}, IRS-1 JNK-binding domain mutant.

**FIG. 7.** Ser\textsuperscript{307} is required for inhibition of IRS-1-dependent insulin-stimulated ERK2 activity by anisomycin. Proteins in whole cell lysates from 32D\textsuperscript{IR}/IRS-1 (A), 32D\textsuperscript{IR}/A307\textsuperscript{IRS-1} (32D\textsuperscript{IR}/IRS\textsuperscript{1}\textsuperscript{307A}) (B), and 32D\textsuperscript{IR} (A and B) cells treated with the indicated doses of anisomycin (Aniso) for 30 min prior to stimulation with 10 nM insulin for 5 min were analyzed with antibodies against phosphorylo-MAPK (pMAPK) and ERK2.
consequent with the general inhibition of tyrosine phosphorylation expected during inhibition of PTB domain function by Ser307 phosphorylation. This general inhibition is in contrast to the specific inhibition of p85 association at tyrosine phosphorylation motifs directly adjacent to previously identified inhibitory serine residues (31, 45, 53).

At least three kinases apparently mediate phosphorylation of Ser307, including a TNF-α/anisomycin-stimulated kinase other than JNK and an insulin/IGF-1-stimulated kinase that is inhibited by wortmannin/LY294002 and requires PI3K activity. We originally thought that JNK might be the common final step that mediates Ser307 phosphorylation downstream of various cytokines, an especially attractive hypothesis since JNK1 and JNK2 apparently mediate phosphorylation of Ser307. These kinases might possess the common ability to bind to the JNK-binding domain in IRS-1, although other mechanisms could be involved.

In summary, potential mediators of chronic insulin resistance, such as TNF-α and hyperinsulinemia, lead to progressive accumulation of IRS-1 molecules that are phosphorylated at Ser307 and that couple less efficiently to the insulin receptor. Chronic Ser307 phosphorylation might also target IRS-1 for degradation or to subcellular compartments inaccessible to the activated insulin receptor. Other IRS proteins, especially IRS-2, might be similarly sensitive to serine phosphorylation. IRS-2 contains a JIP homology region, although a residue analogous to Ser307 does not exist in IRS-2 (20). Nevertheless, IRS-2 is serine-phosphorylated during TNF-α or anisomycin stimulation, which inhibits insulin-stimulated tyrosine phosphorylation. Since IRS-1 is essential to sustain compensatory insulin secretion in mice, serine phosphorylation-mediated inhibition might promote both peripheral insulin resistance and β-cell failure. Identification of the phosphorylation sites in IRS-2 that inhibit insulin-stimulated tyrosine phosphorylation and the kinase specific to those sites is an important target for future mechanism-based drug discovery.

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