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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 IRS[1] 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 phosphoro-specific Akt antibodies were purchased from New England Biolabs Inc. Control anti-Akt and anti-JNK1 antibodies were purchased from Santa Cruz Biotechnology. Anti-phosphotyrosine antibodies were purchased from Transduction Laboratories. Antibodies against IRS-1, IRS-2, and p85 have been described (21, 22). Antibodies directed against phosphorylated Ser^{612} in IRS-1 were purchased from BIOSOURCE. 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.

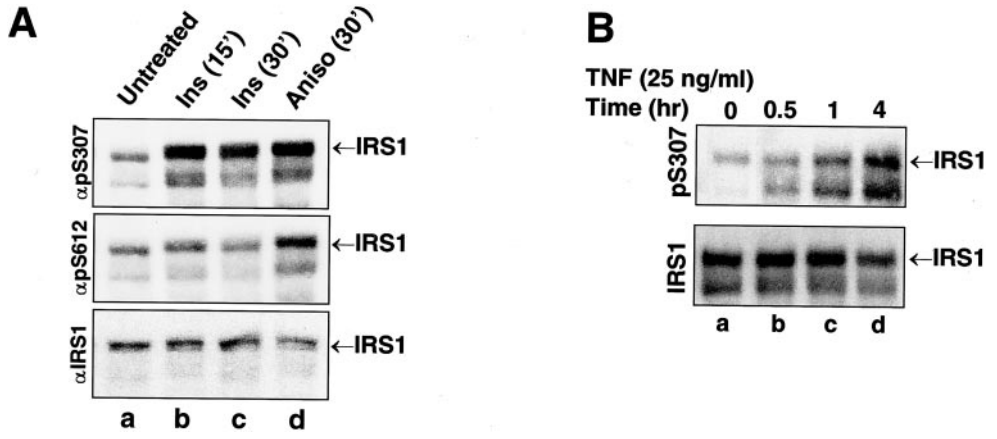

FIG. 1. **Anisomycin and TNF-α induce phosphorylation of Ser^{307} in IRS-1.** *A*, IRS-1 immunoprecipitates from 32D^{IR}/IRS-1 cells treated with 10 nM insulin (*Ins*) or 1.0 μg/ml anisomycin (*Aniso*) for the indicated times were analyzed by immunoblotting with αpS^{307}, αpS^{612}, and anti-IRS-1 antibody. *B*, IRS-1 immunoprecipitates from 32D^{IR}/IRS-1 cells treated with 25 ng/ml TNF-α for the indicated times were analyzed by immunoblotting with αpS^{307} or anti-IRS-1 antibody.

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 transfectants 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, 1.0% Nonidet P-40, 100 mM NaF, 50 mM β-glycerophosphate, 100 μM $NaVO_4$, 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 ^{125}I-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 transfectants 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 $MgCl_2$, 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-^{32}P]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, ^{32}P phosphorylation of substrate proteins was examined by autoradiography and Cerenkov counting. PI3K activity assays were performed on IRS-1 immunoprecipitates as previously described (28).

Yeast Transformation and Interaction Assay—The yeast MATCHMAKER LexA two-hybrid system reagents were purchased from CLONTECH. The yeast *Saccharomyces cerevisiae* strain EGY48 (*Mat*α, *trp1*, *his3*, *ura3*, *6LexAop-LEU2*, *LYS2*) and the pLexA-IR and pB42AD-IRS-1/2 constructs were a generous gift from Thomas A. Gustafson. EGY48 was sequentially transformed with plasmid constructs by the polyethylene glycol/lithium acetate method according to the CLONTECH protocol. Transformants were grown on the appropriate SD/glucose-agar plates for 3 days at 30 °C. Four independent colonies were streaked onto SD/glucose agar plates, grown overnight, replica-plated onto SD/galactose/raffinose agar plates, and regrown for 5 days at 30 °C to induce expression of B42 fusion proteins and to determine interacting partners.

Tri-hybrid Disruption Assay—EGY48 was sequentially transformed as described above with pLexA-IR, various pB42AD-IRS-1 constructs, and a third plasmid (pDIS) containing various cDNAs. Transformants were grown on the appropriate SD/glucose agar plates for 3 days at 30 °C. Four independent colonies were streaked onto SD/glucose agar plates, incubated overnight, and then replica-plated onto SD/galactose/raffinose agar plates. The plates were immediately replica-cleaned, incubated overnight, replica-cleaned, and incubated at 30 °C for 5 days to promote growth and to induce expression of B42 fusion proteins.

RESULTS

Phosphorylation of Ser^{307} 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, Ser^{307} in rat IRS-1, was originally found to be phosphorylated specifically by JNK. Ser^{307} 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 Ser^{307} is interesting because it inhibits insulin-stimulated tyrosine phosphorylation of rat IRS-1. To study the role of Ser^{307} in insulin signaling, full-length rat IRS-1 or a mutant IRS-1 molecule containing a Ser^{307} $\rightarrow$ Ala substitution (A307^{IRS-1}) was stably expressed in 32D^{IR} 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 (28, 30).

Immunoprecipitates of IRS-1 from 32D^{IR} cells stimulated with insulin or anisomycin were analyzed by immunoblotting with phospho-specific antibodies against Ser^{307} (αpS^{307}) and, for comparison, Ser^{612} (αpS^{612}). Before stimulation, both antibodies reacted weakly with IRS-1, indicating that these phosphorylation sites were slightly phosphorylated under the basal conditions (Fig. 1*A*). Anisomycin or insulin strongly stimulated phosphorylation of Ser^{307} and Ser^{612}, whereas Ser^{612} was phosphorylated only during anisomycin stimulation (Fig. 1*A*). As previously shown (20), TNF-α stimulated Ser^{307} phosphorylation more slowly than either insulin or anisomycin (Fig. 1*B*).

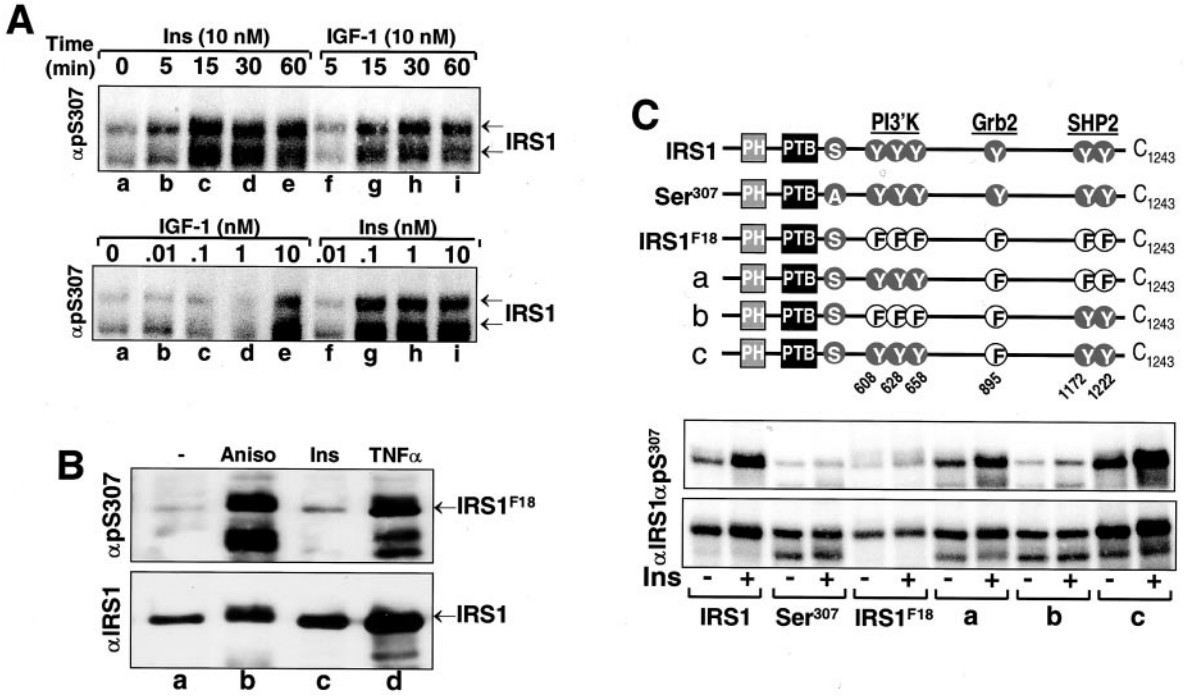

FIG. 2. **Insulin induces Ser^{307} phosphorylation in a PI3K-dependent manner.** *A*, IRS-1 immunoprecipitates from 32D^{IR}/IRS-1 cells treated with 10 nM insulin (*Ins*) or 10 nM IGF-1 for the indicated times were analyzed by immunoblotting with αpS^{307}. IRS-1 immunoprecipitates from 32D^{IR}/IRS-1 cells treated with the indicated doses of insulin and IGF-1 for 30 min were analyzed by immunoblotting with αpS^{307} and anti-IRS-1 antibody. *B*, IRS-1 immunoprecipitates from 32D^{IR}/IRS-1^{F18} cells treated with 5 μg/ml anisomycin (*Aniso*), 10 nM insulin, or 25 ng/ml TNF were analyzed by immunoblotting with αpS^{307} and anti-IRS-1 antibody. *C*, shown is a schematic of various IRS-1 tyrosine phosphorylation site mutants. IRS-1 immunoprecipitates from 32D^{IR}/IRS-1 and 32D^{IR} cells stably expressing various IRS-1 tyrosine phosphorylation site mutants treated with 10 nM insulin for 30 min were analyzed by immunoblotting with αpS^{307} and anti-IRS-1 antibody.

Thus, Ser^{307} is a common phosphorylation site for several signaling pathways that was detected specifically by immunoblotting with αpS^{307}.

Insulin-stimulated Ser^{307} Phosphorylation Requires Tyrosine Phosphorylation of Rat IRS-1—Insulin rapidly stimulated phosphorylation of Ser^{307} in 32D^{IR} cells (Fig. 2*A*). Ser^{307} phosphorylation was more sensitive to insulin than to IGF-1, owing presumably to the higher expression of recombinant insulin receptor in these cells. Previous results showed that PI3K inhibitors block insulin-stimulated Ser^{307} phosphorylation (29). To determine whether insulin-stimulated Ser^{307} phosphorylation requires tyrosine phosphorylation, IRS-1^{F18} was examined in 32D^{IR} cells. IRS-1^{F18} lacks 18 potential tyrosine phosphorylation sites and fails to bind various SH2 proteins, including p85, Grb2, and SHP2 (23). Interestingly, IRS-1^{F18} was not phosphorylated at Ser^{307} during insulin stimulation, whereas it was phosphorylated normally during anisomycin and TNF-α stimulation (Fig. 2*B*). Insulin-stimulated Ser^{307} phosphorylation was restored by site-directed mutagenesis that replaced three PI3K-binding sites in IRS-1^{F18}, including Tyr^{608}, Tyr^{628}, and Tyr^{658}. By contrast, restoring the SHP2-binding sites in IRS-1^{F18} did not promote Ser^{307} phosphorylation. Moreover, deletion of the Grb2-binding motif in IRS-1 did not inhibit insulin-stimulated Ser^{307} phosphorylation (Fig. 2*C*). Thus, several distinct signaling pathways, including insulin stimulation of the PI3K cascade, converge at Ser^{307} to mediate negative feedback or heterologous inhibition of IRS-1 signaling to inhibit the insulin response.

Ser^{307} Phosphorylation Inhibits IR/IRS-1 Interaction—During experiments in mammalian cells and yeast, the PTB domain of IRS-1 binds to the phosphorylated NPEY motif in the juxtamembrane region of the insulin receptor (32–35). In mammalian cells, this interaction promotes efficient phosphorylation of IRS-1 during insulin stimulation. Since Ser^{307} 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 Ser^{307} 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 orthologs) that might specifically bind JNK1 (Fig. 3*A*) (20, 36). The L*X*L sequence of this motif in JIP-1 is required for JNK interaction (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 construct lacking both JIP homology regions did not promote yeast growth (Fig. 3*B*). IRS-1 constructs retaining residues 516–865 or 822–888, which contain the JIP homology region in the overlapping sequence, promoted growth and β-galactosidase activity (Fig. 3*B*). 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 (RPTRLSL^{858} 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. 3*C*). How-

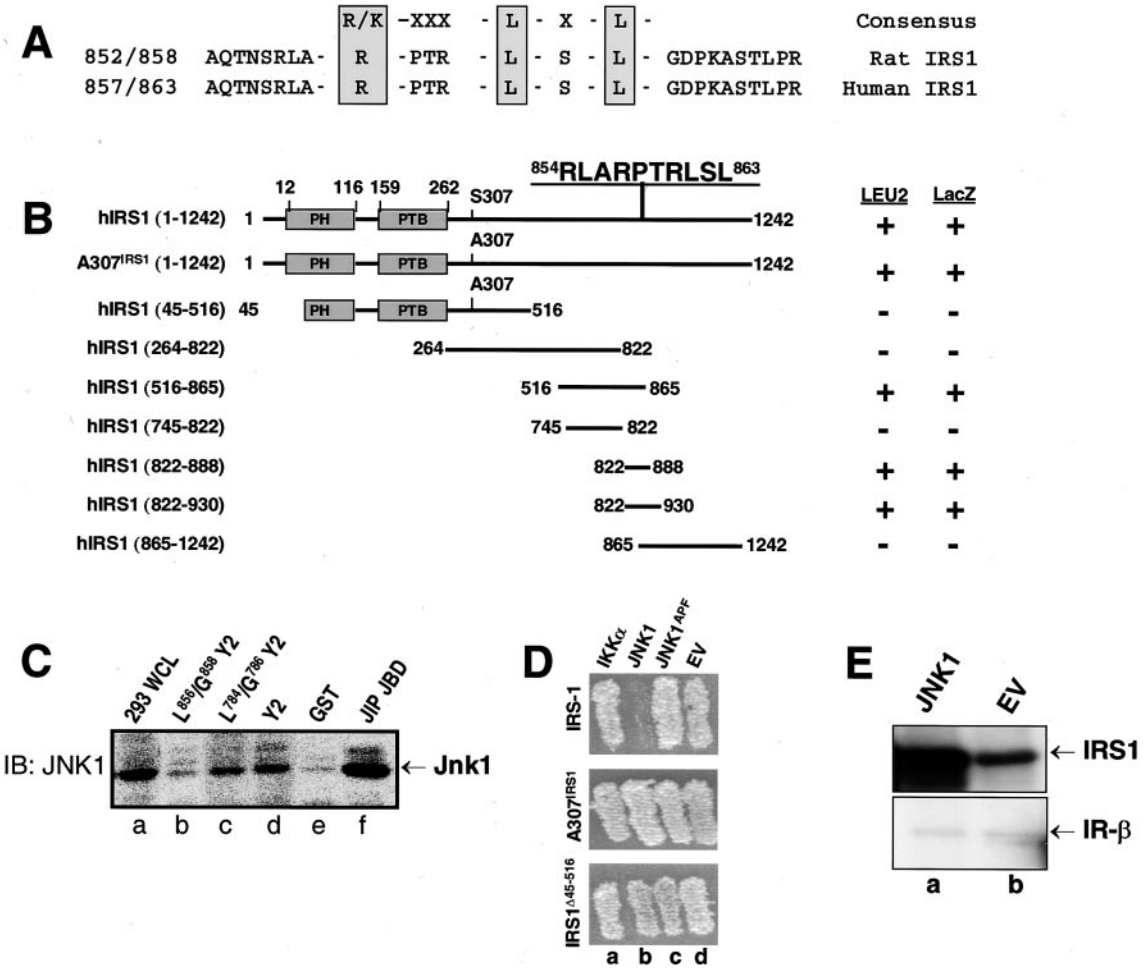

FIG. 3. **Ser^{307} phosphorylation inhibits insulin action through disruption of the IR/IRS-1 interaction.** *A*, shown is a schematic comparing the JNK-binding domain at residues 856–858 in the rat IRS-1 sequence and residues 861–863 in the human IRS-1 sequence. *B*, shown is a schematic representation from the yeast interaction analysis of human JNK1 (bait) and various truncations of human IRS-1 (*hIRS1*; prey). Symbols are representative of the presence (+) or lack (−) of interaction between given partners, as determined by growth on selective medium (using two independent reporters, *LEU2* and *lacZ*). *C*, the JNK-binding domain of JIP-1 (*JIP JBD*), the Y2 region (amino acids 555–898) of IRS-1, the Y2 region of IRS-1 in which the pair of leucines at positions 856 and 858 in the potential JNK-binding domain were mutated to glycine, and the Y2 region of IRS-1 in which the pair of leucines at positions 784 and 786 in the potential JNK-binding domain were mutated to glycine were expressed as GST fusion proteins (111 pmol) and incubated with HEK293 whole cell lysates (*WCL*). Whole cell lysates and GST pull-down assays were analyzed by immunoblotting (*IB*) with antibodies against JNK1. *D*, shown are the results from yeast tri-hybrid disruption analysis of the interaction between the human insulin receptor (bait), human IRS-1 (prey), and human A312^{IRS-1} (prey) by human JNK1 (disruptant). The IR/IRS-1 (bait/prey) interaction was scored by growth on selective medium. Disruption of the IR/IRS-1 interaction by JNK1, catalytically inactive JNK1 (JNK1^{APF}), or an empty vector control (*EV*) was assayed as a lack of growth on selective medium. *E*, IRS-1 and baculovirus-expressed insulin receptor (*IR-β*) were subjected to *in vitro* kinase assay with JNK1 expressed and activated with 5.0 μg/ml anisomycin in HEK293 cells. HEK293 cells transfected with empty vector were assayed in parallel.

ever, a Leu$^{852/858}$ → Gly substitution, but not a Leu$^{784/786}$ → Gly substitution, abolished the ability of rat IRS-1 to pull-down JNK1 (Fig. 3*C*).

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^{APF}) or an empty vector was expressed in these yeast cells (Fig. 3*D*). By contrast, coexpression of active JNK1 prevented growth, suggesting that the IR/IRS-1 interaction was inhibited (Fig. 3*D*). Substitution of Ser^{312} for alanine in human IRS-1 (orthologous to Ser^{307} in rat IRS-1) blocked JNK1-mediated growth inhibition, suggesting that JNK1-mediated phosphorylation of Ser^{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^{312} but lacking the JIP homology region, was insensitive to JNK1-mediated disruption of the IR/IRS-1 interaction (Fig. 3*D*). The insulin receptor did not interact with JNK1 in a yeast two-hybrid assay (data not shown) and was not phosphorylated by JNK1 during *in vitro* kinase assays using purified insulin receptor and JNK1 (Fig. 3*E*). These results are consistent with the hypothesis that the interaction between the insulin receptor and IRS-1 is inhibited by phosphorylation of Ser^{307} during association of JNK1 with the JIP homology region of IRS-1.

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

Tyrosine phosphorylation of IRS-1 was detected by immunoblotting with anti-phosphotyrosine antibodies as previously described (37). Treatment of 32D^{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. 5*A*). Low concentrations of anisomycin had no inhibitory effect on tyrosine phosphorylation of A307^{IRS-1}, and inhibition barely reached 15% at 1.0 μg/ml anisomycin (Fig. 5*A*). 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 L*X*L sequence of the JNK-binding domain of JIP-1 to G*X*G abrogates JNK interaction (26).[2] Inactivation of the JIP homology region by point mutations of the L^{856}SL motif to a G^{856}SG 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 Ser^{307}.

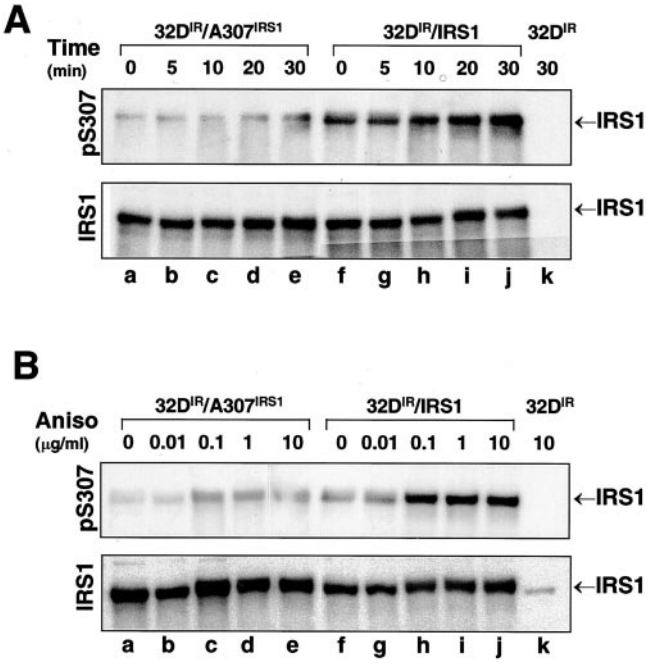

FIG. 4. **Anisomycin induces phosphorylation of Ser^{307} in IRS-1 in a dose- and time-dependent manner.** *A*, IRS-1 immunoprecipitates from 32D^{IR}, 32D^{IR}/IRS-1, and 32D^{IR}/A307^{IRS-1} cells treated with 1.0 μg/ml anisomycin for the indicated times were analyzed by immunoblotting with αpS^{307} and anti-IRS-1 antibody. *B*, IRS-1 immunoprecipitates from 32D^{IR}, 32D^{IR}/IRS-1, and 32D^{IR}/A307^{IRS-1} cells treated with the indicated doses of anisomycin (*Aniso*) for 30 min were analyzed by immunoblotting with αpS^{307} and anti-IRS-1 antibody.

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 32D^{IR} cells; however, the binding of A307^{IRS-1} to p85 during insulin stimulation was not inhibited by anisomycin (Fig. 5*B*). 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 A307^{IRS-1} during insulin stimulation (Fig. 5*C*).

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 32D^{IR} cells, analysis of the inhibitory effect of anisomycin on ERK1 phosphorylation was confounded by stimulation of ERK1 phosphorylation by anisomycin and insulin

[2] R. J. Davis, personal communication.

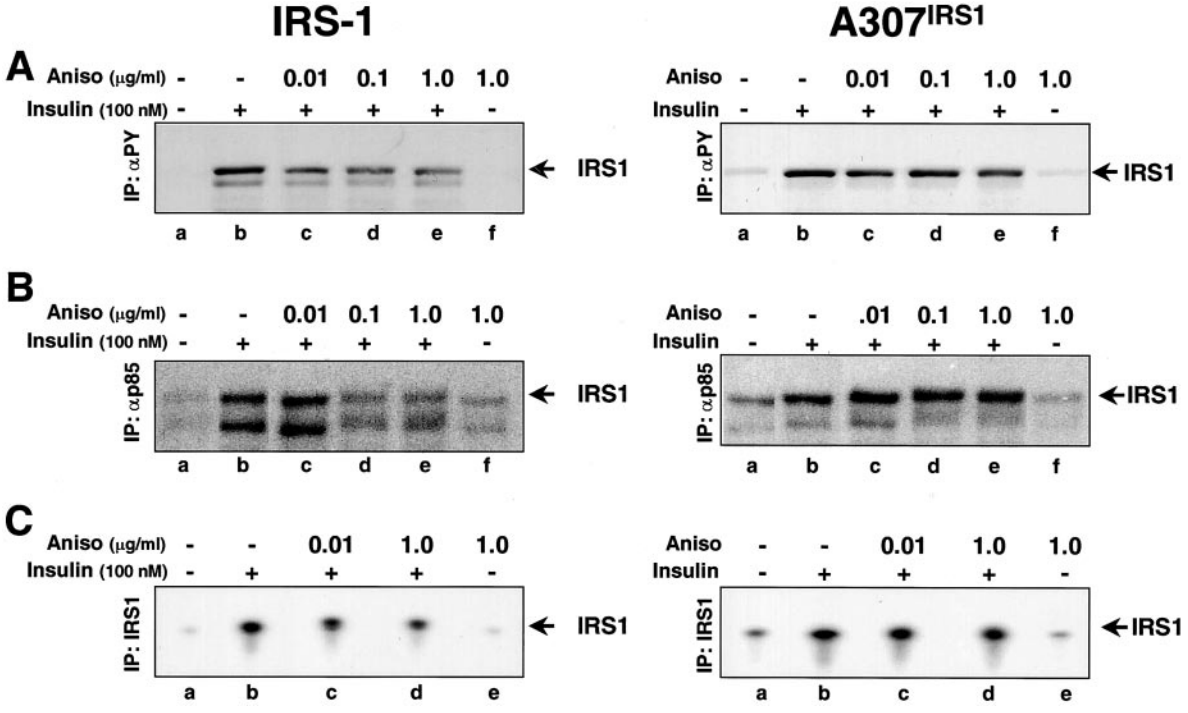

FIG. 5. **Ser^{307} in IRS-1 is required for inhibition of insulin signaling by anisomycin.** *A*, proteins in whole cell lysates from 32D^{IR}/IRS-1 and 32D^{IR}/A307^{IRS-1} cells treated with the indicated doses of anisomycin (*Aniso*) for 30 min prior to stimulation with 10 nM insulin for 5 min were analyzed with anti-phosphotyrosine antibodies (α*PY*). *B*, immunoprecipitates (*IP*) of the p85 regulatory subunit of PI3K from 32D^{IR}/IRS-1 and 32D^{IR}/A307^{IRS-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 32D^{IR}/IRS-1 and 32DIR/A307^{IRS-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.

through the Shc pathway in the absence of IRS-1 or A307^{IRS-1} expression (Fig. 7*A*). By contrast, ERK2 was phosphorylated only during insulin stimulation of 32D^{IR} cells expressing either IRS-1 or A307^{IRS-1} (Fig. 7, *A* and *B*). Anisomycin completely inhibited insulin stimulation of ERK2 phosphorylation (60% inhibition at 0.1 μg/ml) in 32D^{IR}/IRS-1 cells. By contrast, ERK2 phosphorylation was barely inhibited (20%) at the highest anisomycin concentration in 32D/A307^{IRS-1} cells (Fig. 7*B*). These data reveal that phosphorylation of Ser^{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^{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^{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^{312} → 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^{307} phosphorylation completely abrogates insulin receptor/IRS-1 interaction, the 32D^{IR} cell-based experiments suggest that it inhibits IRS-1 tyrosine phosphorylation by only 50% at best.

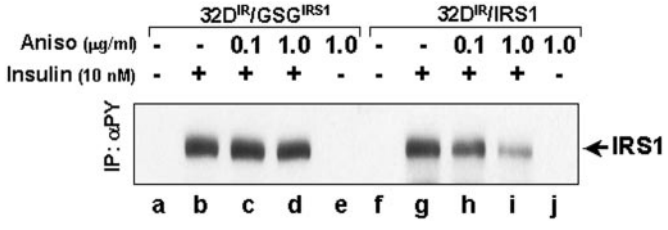

FIG. 6. Proteins in whole cell lysates from 32D^{IR}/IRS-1 and 32D^{IR}/GSG^{IRS-1} cells treated with the indicated doses of anisomycin (*Aniso*) for 30 min prior to stimulation with 10 nM insulin for 5 min were analyzed with anti-phosphotyrosine antibodies (*αPY*). *IP*, immunoprecipitate *GSG^{IRS-1}*,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

Previous work revealed that efficient phosphorylation of IRS-1 depends on two N-terminal domains, the pleckstrin homology (PH) domain and the adjacent PTB domain (35, 40). Deletion of both the PH and PTB domains completely inhibits phosphorylation during insulin stimulation of 32D^{IR} cells, whereas deletion of either the PH or PTB domain partially reduces tyrosine phosphorylation. Since Ser^{307} phosphorylation inhibits PTB domain function, persistent coupling mediated through the PH domain might be responsible for incomplete inhibition of IRS-1 tyrosine phosphorylation. Efficient coupling of IRS-1 to low levels of insulin receptors requires both domains, whereas either the PH or PTB domain is sufficient in cells expressing high levels of insulin receptor. Therefore, in cells with a low number of receptors, Ser^{307} phosphorylation might play a major regulatory role, whereas Ser^{307} phosphorylation might be inefficient in cells with a high number of receptors (35). Under the latter condition, more drastic regulatory mechanisms might be required, including degradation of IRS-1.

Considerable evidence is largely consistent with the hypothesis that serine phosphorylation of the insulin receptor or the IRS proteins inhibits signal transduction. Despite the potential importance of this regulatory pathway, the sites of phosphorylation and the inhibitory mechanisms involved have been difficult to identify. Increased serine phosphorylation of IRS-1 is a common finding in insulin resistance and type II diabetes (41). Serine-phosphorylated IRS-1 inhibits insulin-stimulated autophosphorylation of the insulin receptor, PI3K activation, glucose uptake, and other insulin-stimulated biological responses (3, 42–50). Besides the JNK phosphorylation site at Ser^{307}, IRS-1 contains serine/threonine residues in consensus sequences for many other protein kinases, including casein kinase II, cAMP-dependent protein kinase, protein kinase C, Cdc2 kinase, MAPK, and protein kinase B/Akt (21, 42, 44, 51–53). Recent reports suggest that serine phosphorylation of IRS-1 inhibits its ability to associate with the insulin receptor and to serve as a substrate for tyrosine phosphorylation (3, 42, 45, 50, 53, 54). Thus, the identification of serine/threonine phosphorylation-based mechanisms of signal inhibition might reveal a molecular basis for insulin resistance that promotes the pathogenesis of type II diabetes.

Ser^{307} phosphorylation promotes general inhibition of IRS-1 signaling, as revealed by reduced activation of both the PI3K and MAPK cascades. This effect does not occur through inhibition of insulin receptor autophosphorylation, but is consistent with reduced coupling between the insulin receptor and IRS-1. Association of p85 or Grb2 with IRS-1 depends on distinct sets of tyrosine phosphorylation motifs that are separated by up to 300 amino acids in the primary sequence. These results are

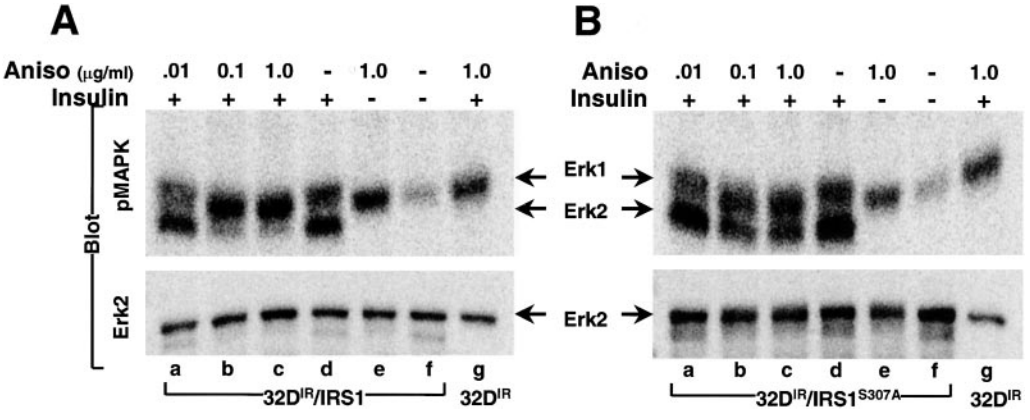

FIG. 7. **Ser^{307} is required for inhibition of IRS-1-dependent insulin-stimulated ERK2 activity by anisomycin.** Proteins in whole cell lysates from 32D^{IR}/IRS-1 (*A*), 32D^{IR}/A307^{IRS-1} (*32D^{IR}/IRS1^{S307A}*) (*B*), and 32D^{IR} (*A* and *B*) cells treated with the indicated doses of anisomycin (*Aniso*) for 30 min prior to stimulation with 10 nM insulin for 5 min were analyzed with antibodies against phospho-MAPK (*pMAPK*) and ERK2.

consistent with the general inhibition of tyrosine phosphorylation expected during inhibition of PTB domain function by Ser^{307} 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 Ser^{307}, 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 Ser^{307} phosphorylation downstream of various cytokines, an especially attractive hypothesis since JNK1 binds to IRS-1 (20) and since the JNK-binding region of IRS-1 is required for the inhibition of insulin-stimulated tyrosine phosphorylation of IRS-1 by anisomycin. However, some experiments with potential physiological mediators of insulin resistance do not support this hypothesis. Whereas anisomycin and TNF-α stimulate JNK and Ser^{307} phosphorylation, the MEK kinase inhibitor PD98059 completely inhibits Ser^{307} phosphorylation, with no effect on JNK activity (29). Although insulin activates JNK in certain cells, this pathway is not inhibited by wortmannin/LY294002, suggesting that a distinct cascade is involved. Therefore, in addition to JNK, at least two other kinases apparently mediate phosphorylation of Ser^{307}. 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 Ser^{307} and that couple less efficiently to the insulin receptor. Chronic Ser^{307} 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 Ser^{307} 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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