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The c-Jun NH_2-terminal Kinase Promotes Insulin Resistance during Association with Insulin Receptor Substrate-1 and Phosphorylation of Ser^{307}*

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Tumor necrosis factor α (TNFα) inhibits insulin action, in part, through serine phosphorylation of IRS proteins; however, the phosphorylation sites that mediate the inhibition are unknown. TNFα promotes multipotential signal transduction cascades, including the activation of the Jun NH_2-terminal kinase (JNK). Endogenous JNK associates with IRS-1 in Chinese hamster ovary cells. Anisomycin, a strong activator of JNK in these cells, stimulates the activity of JNK bound to IRS-1 and inhibits the insulin-stimulated tyrosine phosphorylation of IRS-1. Serine 307 is a major site of JNK phosphorylation in IRS-1. Mutation of serine 307 to alanine eliminates phosphorylation of IRS-1 by JNK and abrogates the inhibitory effect of TNFα on insulin-stimulated tyrosine phosphorylation of IRS-1. These results suggest that phosphorylation of serine 307 might mediate, at least partially, the inhibitory effect of proinflammatory cytokines like TNFα on IRS-1 function.

The insulin signaling system is complex, and a common mechanism to explain the occurrence of insulin resistance during diabetes is difficult to resolve. So far, genetic approaches provide important insight into certain early onset forms of diabetes but fail to explain insulin resistance that is associated with common type 2 diabetes (1–4). Recent results support the notion that dysregulation of the insulin receptor and reduced tyrosine phosphorylation of the IRS[1] proteins might contribute significantly to peripheral insulin resistance and β-cell failure (5, 6).

The principal insulin receptor substrates, IRS-1 and IRS-2, are phosphorylated on multiple tyrosine residues by the activated receptors for insulin, IGF-1, and various other cytokines (7). Tyrosine phosphorylation of IRS-1 and IRS-2 promotes their binding to the Src homology 2 domains in various downstream signaling proteins, including the phosphatidylinositol 3-kinase (PI 3-kinase), Grb-2, SHP2, and others (7, 8). During association with IRS proteins, PI 3-kinase is activated, and its phospholipid products promote the recruitment of various serine kinases to the plasma membrane, where they are activated by phosphorylation (9). One of the membrane-associated kinases, protein kinase B/Akt, phosphorylates multiple downstream effectors that promote diverse biological responses, including stimulation of glucose transport, protein and glycogen synthesis, and the regulation of gene expression, which affects cellular proliferation and survival (10, 11).

The insulin receptor and the IRS proteins might be counterregulated by degradation, differential expression, or modification by serine/threonine phosphorylation (12–15). Increased serine phosphorylation of IRS-1 is a common finding during insulin resistance and type 2 diabetes (16). However, the mechanism by which serine phosphorylation inhibits insulin signaling is difficult to establish, because IRS-1 contains more than 70 potential serine/threonine residues in consensus sequences for many protein kinases, including casein kinase II, cAMP-dependent protein kinase, protein kinase C, Cdc2 kinase, MAP kinase, and protein kinase B/Akt (14, 15, 17–20).

The action of proinflammatory cytokines like TNFα or interleukin-1β to promote serine phosphorylation of IRS-1 might provide a common mechanism for insulin/IGF-1 resistance observed during acute trauma and chronic obesity (21–26). TNFα is produced systemically by macrophages and lymphocytes after inflammatory stimulation or trauma and increases rapidly during experimental injury induced by cerebral ischemic, excitotoxic, and traumatic injury (27). Moreover, obese animals and humans also produce TNFα in positive correlation to body mass index and hyperglycemia, an indirect measure of insulin resistance (23, 24). During chronic obesity or sudden trauma, insulin receptor kinase activity and tyrosine phosphorylation of IRS-1 are reduced in skeletal muscle; however, in each case dephosphorylation of IRS-1 by incubation *in vitro* with alkaline phosphatase restores its ability to undergo tyrosine phosphorylation by the activated insulin receptor (28–30). TNFα receptor null mice display less insulin resistance during diet-induced obesity, suggesting that TNFα signals promote, at least partially, the inhibition of IRS-1 function in mice (31, 32). Moreover, TNFα treatment of adipocytes increases serine phosphorylation of IRS-proteins, which inhibits insulin-stimulated tyrosine phosphorylation and impairs insulin signaling (28, 29, 34). These results suggest that serine phosphorylation of IRS-1 promotes an inhibitory effect of proinflammatory cytokines on insulin receptor signaling (30).

The identification of the serine kinases that phosphorylate

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[1] The abbreviations used are: IRS, insulin receptor substrate; JNK, c-Jun NH_2-terminal kinase; MAP, mitogen-activated protein; MAPKK, MAP kinase kinase; MAPKKK, MAPKK kinase; TNFα, tumor necrosis factor; CHO, Chinese hamster ovary; PAGE, polyacrylamide gel electrophoresis; GST, glutathione S-transferase; HPLC, high pressure liquid chromatography; PI, phosphatidylinositol; JIP, JNK-interacting protein; JBD, JNK-binding domain; IVK, *in vitro* kinase; IP, immunoprecipitate(s); PTB, phosphotyrosine binding domain.

IRS proteins during acute trauma and chronic obesity is an essential step for learning how to reverse the insulin resistance that perturbs metabolic homeostasis and contributes to diabetes. The JNK signaling pathway is implicated in many biological responses, including mammalian embryogenesis and the response to stress (35, 36). During TNFα binding, the tumor necrosis factor receptor 1 trimerizes, which promotes the assembly of a multicomponent complex that activates the JNK signaling cascade (37). Activated JNK phosphorylates many cellular proteins, including components of the AP-1 transcription factor complex (38). Here we provide evidence to support the hypothesis that JNK associates with IRS-1 and promotes the phosphorylation of Ser^{307} near the PTB domain. Our results suggest that phosphorylation of Ser^{307} by JNK or another related kinase might mediate the inhibitory effects of TNFα on insulin signal transduction.

MATERIALS AND METHODS

Antibodies and Reagents—Phosphospecific antibodies were purchased from New England Biolabs. Antibodies against IRS-1, IRS-2, and p85 were described previously (39, 40). JNK1 antibody was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), and rabbit polyclonal antibodies against p38 have been described (41). Insulin, alkaline phosphatase, M2 antibody, and phosphoamino standards were purchased from Sigma. Constructs for JNK1, GST-ATF2, and GST-JIP-1-JBD have been described (42–44). Point mutants in IRS-1 were generated using the Stratagene Quikchange site-directed mutagenesis method.

Cell Culture—Chinese hamster ovary (CHO) cells overexpressing the human insulin receptor were described previously (45, 46). Stable CHO cell lines expressing murine IRS-1 or murine IRS-2 were generated by Fugene-6-mediated transfection of pCMVHis containing the appropriate inserts (Roche Molecular Biochemicals); 32D cell transfectants were generated by electroporation. In each case, transfected cells were selected in histidinol as described previously (47). CHO cells were maintained in Ham's F-12 medium supplemented with 10% fetal bovine serum and 5 mM histidinol and made quiescent by serum starvation for 12 h, whereas 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. HEK 293 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—CHO and 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 and collected on protein A-Sepharose. Lysates and immunoprecipitates were resolved by SDS-PAGE and transferred to nitrocellulose, and proteins were detected by immunoblotting/^{125}I-labeled protein A and analysis on a Molecular Dynamics PhosphorImager. 293 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.

Alkaline Phosphatase Treatment—Cell lysates from CHO^{IR}/IRS-1 cells treated with anisomycin were incubated with 20 units of alkaline phosphatase at 37 °C for 1 h. The dephosphorylation reaction was stopped by the addition of SDS sample buffer and boiling. Proteins were resolved by SDS-PAGE, transferred to nitrocellulose, and analyzed by Western blot using antibodies against IRS-1(47).

Association of IRS-1 with JNK or p38—GST fusion proteins containing portions of IRS-1 were made by subcloning the indicated residues into pGex-2TK (Amersham Pharmacia Biotech), expressed in *Escherichia coli* (BL-21) and purified using glutathione-agarose (Amersham Pharmacia Biotech). GST fusion proteins (111 pmol) were incubated with 293 cell lysates for 2 h at 4 °C. Where indicated, experiments were performed in the presence or absence of a 64 μg/ml concentration of a wild type JIP-1-JBD (residues 148–174) synthetic peptide or a random sequence control peptide (48). Proteins bound to the GST fusion proteins were fractionated by SDS-PAGE, transferred to nitrocellulose, and analyzed by Western blot with antibodies against JNK1 or p38 (48).

In Vitro Protein Kinase Assay—HEK 293 cells were transiently transfected with either pCDNA3-FLAG-JNK1 or pCNDA3 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 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. IRS-1 was immunopurified from quiescent CHO^{IR}/IRS-1 cells. Kinase assays were initiated by the addition of kinase and 50 μM [γ-^{32}P]ATP to IRS-1 immune complexes, 1 mg of $ATF2^{GST}$, or 1 mg of NH_2-Jun^{GST} in a final volume of 50 μl of kinase buffer. Where indicated, kinase assays were performed in the presence of 10 μM LY294002 (Calbiochem). Reactions were terminated after 30 min at room temperature with ice-cold PBS and the addition of SDS-sample buffer. Phosphorylation of substrate proteins was examined, after SDS-PAGE and transfer to nitrocellulose, by autoradiography and Cerenkov ^{32}P counting.

Biochemical Analysis of IRS-1 Phosphorylation—Metabolic labeling of CHO cells with [^{32}P]orthophosphate was performed as described (49). Tryptic peptides were generated from IRS-1 on nitrocellulose and resolved on a Waters HPLC system equipped with a Hi-Pore reverse phase column (Bio-Rad) as described (49, 50). Phosphoamino acid analysis and manual radiosequencing by Edman degradation were performed as described (49, 50). Endoproteinase Glu-C (Promega) digestion of the initial tryptic HPLC peak was performed in 100 mM ammonium bicarbonate (pH 7.8) for 24 h at 37 °C.

RESULTS

JNK-1 Stimulates Serine Phosphorylation and Inhibits Insulin-stimulated Tyrosine Phosphorylation of IRS-1—TNFα promotes many biological responses, including the activation of the c-Jun NH_2-terminal kinase (JNK) and p38 MAP kinase, which might mediate the phosphorylation of IRS-1 (38, 41). To investigate the phosphorylation of IRS-proteins by these kinases, we activated JNK and p38 in Chinese hamster ovary cells expressing the insulin receptor (CHO^{IR}) and either IRS-1 or IRS-2. Anisomycin is a protein synthesis inhibitor, but at low concentrations it strongly activates JNK and p38 without inhibition of protein synthesis (52, 53). CHO^{IR}/IRS-1 cells were treated for 30 min with anisomycin, and cell lysates were resolved by SDS-PAGE and screened with phosphospecific antibodies against c-Jun (a JNK1 substrate) and p38. Anisomycin induced the phosphorylation of each protein, confirming that JNK and p38 were activated by anisomycin in CHO^{IR}/IRS-1 cells (Fig. 1*A*). In addition, anisomycin reduced the rate of migration of IRS-1 during SDS-PAGE, and alkaline phosphatase treatment of cell lysates restored a normal rate of migration (Fig. 1*B*). These results support the hypothesis that anisomycin stimulates phosphorylation of IRS-1 with kinetics similar to those observed for the activation of JNK and p38.

The effect of anisomycin on insulin-stimulated tyrosine phosphorylation was investigated by immunoblotting CHO^{IR} cell extracts expressing IRS-1 or IRS-2 with antibodies against phosphotyrosine. Before incubation with anisomycin, insulin (25 nM) strongly increased the tyrosine phosphorylation of IRS-1 and IRS-2, whereas treatment of these cells with anisomycin for 30 min before insulin stimulation inhibited tyrosine phosphorylation (Fig. 1, *C* and *D*). Half-maximal inhibition occurred at 1 μg/ml anisomycin; inhibition was associated with a reduced migration of IRS-1 and IRS-2 during SDS-PAGE (Fig. 1, *C* and *D*). As expected, anisomycin inhibited the association of p85 with IRS-1 during insulin stimulation (Fig. 1*E*). Thus, anisomycin appears to promote serine phosphorylation of IRS-1 and IRS-2, which is associated with inhibition of insulin-stimulated tyrosine phosphorylation.

The PTB Domain Is Required for the Inhibitory Effect of Anisomycin on IRS-1 Tyrosine Phosphorylation—IRS-1 contains over 70 putative serine phosphorylation sites and is extensively serine-phosphorylated in the basal state (45). Consequently, the identification of serine phosphorylation sites that

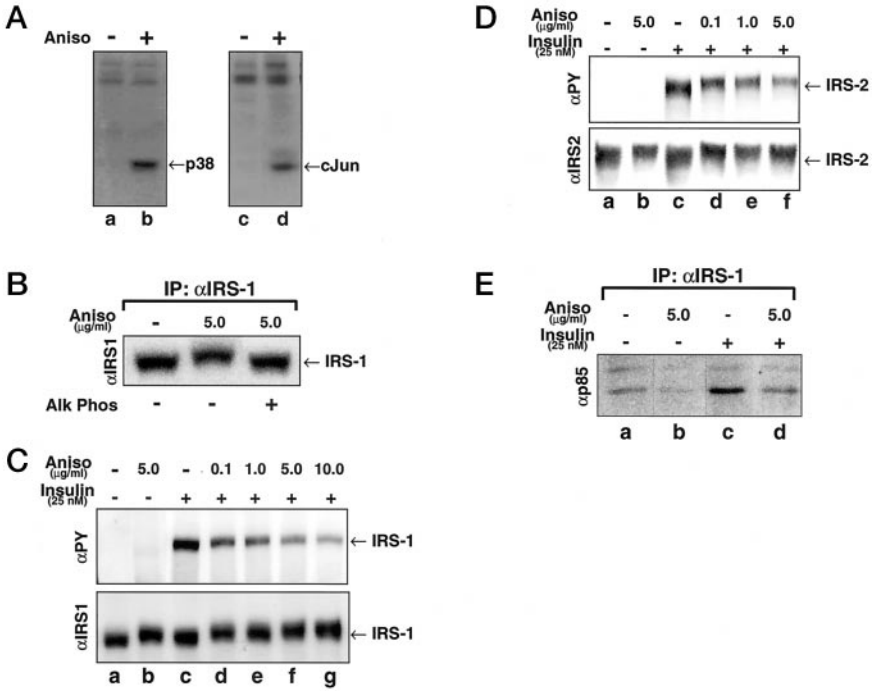

FIG. 1. **Anisomycin inhibits insulin-stimulated tyrosine phosphorylation of IRS proteins.** *A*, CHO^{IR}/IRS-1 cells were treated with (+) or without (−) 5.0 μg/ml anisomycin (*Aniso*) for 30 min, and lysates were analyzed by immunoblotting with phosphospecific antibodies against p38 (*left*) and c-Jun (*right*). *B*, CHO^{IR}/IRS-1 cells were treated with or without 5.0 μg/ml anisomycin for 30 min, and lysates were incubated at 37 °C with (+) or without (−) 20 units of alkaline phosphatase (*Alk Phos*). The reaction was terminated with SDS-sample buffer, and lysates were analyzed by immunoblotting with antibodies against IRS-1. The effect of anisomycin on insulin-stimulated tyrosine phosphorylation of IRS-1 (*C*) and IRS-2 (*D*) is shown. CHO^{IR}/IRS-1 and CHO^{IR}/IRS2 cells were treated for 30 min with or without the indicated concentrations of anisomycin prior to stimulation with or without insulin. Lysates were analyzed by immunoblotting with antibodies against phosphotyrosine (*αPY*), IRS-1, and IRS-2. *E*, CHO^{IR}/IRS-1 cells were treated for 30 min with (*lanes b* and *d*) or without (*lanes a* and *c*) 5.0 μg/ml anisomycin prior to stimulation with (*lanes c* and *d*) or without (*lanes a* and *b*) insulin. IRS-1 immunoprecipitates were analyzed by immunoblotting with antibodies against p85.

inhibit tyrosine phosphorylation is difficult. To identify the regions of IRS-1 that might be involved, various IRS-1 deletion mutants were expressed in CHO^{IR} cells, and the effect of anisomycin on their migration during SDS-PAGE and tyrosine phosphorylation was determined (Fig. 2*A*). The effect of anisomycin (5 μg/ml) to reduce the migration of IRS-1 during SDS-PAGE and to inhibit insulin-stimulated tyrosine phosphorylation was retained upon deletion of the pleckstrin homology domain or deletion of residues 584–898 in the COOH terminus of IRS-1. By contrast, deletion of the PTB domain (residues 140–309) significantly reduced these effects of anisomycin (Fig. 2*B*). Based on this analysis, a truncated IRS-1 protein was constructed, which contained the first 309 residues of IRS-1 (the pleckstrin homology and PTB domains) fused to residues 555–898 of the COOH-terminal tail (Fig. 2*A*). This construct, called PPY^{IRS-1}, is tyrosine-phosphorylated during insulin stimulation, binds PI 3-kinase, and mediates the expected downstream signals (54). Importantly, anisomycin decreased the electrophoretic mobility of PPY^{IRS-1} and inhibited its insulin-stimulated tyrosine phosphorylation, suggesting that PPY^{IRS-1} contains the elements by which anisomycin inhibits IRS-1 tyrosine phosphorylation (Fig. 3). Furthermore, TNFα inhibits the insulin-stimulated tyrosine phosphorylation of PPY^{IRS-1}, but not the phosphorylation of an IRS-1 molecule deleted for the PTB domain, in 32D cells (data not shown).

JNK Associates with PPY^{IRS-1} in CHO^{IR} Cells—In several cases, protein phosphorylation by various MAP kinases is dependent on allosteric binding of the kinase to its substrate or an associated scaffold protein (55). Similarities between the binding motifs of several JNK substrates reveals a putative consensus binding motif defined by the sequence (R/K)*XXXX*-L*X*L (Fig. 4*A*) (44, 56). Interestingly, the IRS-1 primary sequence contains 14 similar motifs, 11 of which are found in the COOH terminus, including two likely sites beginning at Arg^{852} and Arg^{780} (Fig. 4*A*). Since PPY^{IRS-1} retains these putative JNK-binding domains, PPY^{IRS-1} was immunoprecipitated from quiescent CHO^{IR}/PPY^{IRS-1} cells and analyzed by immunoblotting with antibodies against JNK1. JNK1 was detected in PPY^{IRS-1} immunoprecipitates but not in preimmune complexes, suggesting that JNK1 associated with PPY^{IRS-1} *in vivo* (Fig. 4*B*). By contrast, JNK1 did not associate with PP^{IRS-1}, a truncated IRS-1 molecule consisting of only the pleckstrin homology and PTB domains (Fig. 4*B*). Immunoblots with p38-specific antibodies revealed that PPY^{IRS-1} and PP^{IRS-1} did not associate with p38. Furthermore, an inhibitor of p38 activity (SB 230580 (58)) did not block the inhibitory effect of anisomycin on insulin-stimulated tyrosine phosphorylation of IRS-1 (data not shown). Thus, JNK but not p38, might bind to an (R/K)*XXXX*L*X*L motif between amino acids residues 555 and 898 of IRS-1 and mediate serine phosphorylation.

Association and Phosphorylation of IRS-1 with JNK1—The location of the preferred JNK binding region in IRS-1 was further verified with GST fusion proteins containing various regions of IRS-1, including residues 1–309, 140–581, 555–898, or 1064–1235; the JNK-binding domain in JNK-interacting protein (JIP)-1 was used as a positive control in these experiments, since it was shown previously to strongly bind JNK (48). HEK 293 cell lysates were incubated with these immobilized GST fusion proteins, and the binding of JNK1 to these proteins was detected by specific immunoblotting with antibodies against JNK1. Consistent with previous reports, JNK1 strongly bound to the JIP fragment; however, JNK also associated specifically with residues 555–898 of IRS-1, whereas it failed to bind to GST or GST fusion proteins containing other regions of IRS-1 (Fig. 4*C*). The binding of JNK1 was blocked by a synthetic peptide that corresponds to the JNK-binding do-

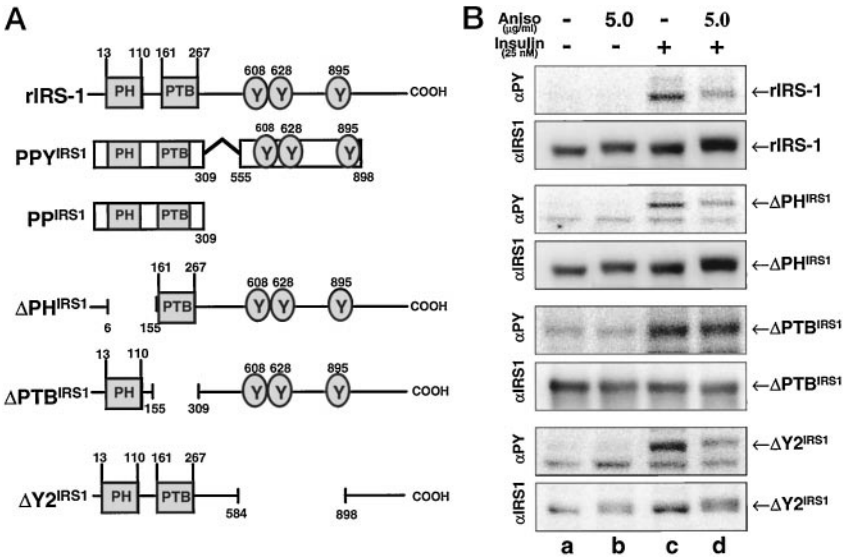

FIG. 2. **The PTB domain is required for the inhibitory effect of anisomycin on IRS-1 tyrosine phosphorylation.** *A*, schematic diagram depicting wild type rat IRS-1 (*rIRS-1*) and various IRS-1 truncation mutants. *B*, the indicated IRS-1 deletion mutants were treated with (+) or without (−) 5.0 μg/ml anisomycin for 30 min prior to stimulation with or without insulin. Lysates were analyzed by immunoblotting with antibodies against phosphotyrosine (*αPY*) and IRS-1.

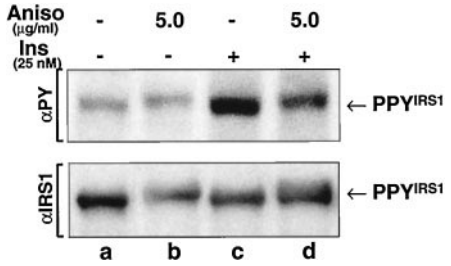

FIG. 3. **PPY^{IRS-1} contains the elements necessary for the inhibition of insulin-stimulated tyrosine phosphorylation by anisomycin.** Insulin-stimulated tyrosine phosphorylation of PPY^{IRS-1} is inhibited by anisomycin. CHO^{IR}/PPY^{IRS-1} cells were treated for 30 min with (*lanes b* and *d*) or without (*lanes a* and *c*) 5.0 μg/ml anisomycin prior to stimulation with (*lanes c* and *d*) or without (*lanes a* and *b*) insulin. Lysates were analyzed by immunoblotting with antibodies against phosphotyrosine (*αPY*) and IRS-1.

main of JIP-1 (Fig. 4*D*).

The activity of JNK associated with IRS-1 was determined in immunocomplexes prepared from cell lysates before and after anisomycin treatment (Fig. 4*E*). The assays were conducted in the presence of the PI 3-kinase inhibitor LY294002 to eliminate background activities due to PI 3-kinase-dependent kinases (14, 59). During incubation of the αIRS-1 immunocomplexes with [^{32}P]ATP, serine phosphorylation of IRS-1 was significantly greater in samples from anisomycin-treated cells, suggesting that JNK might be involved in IRS-1 phosphorylation. To verify that JNK activity associated with IRS-1 was activated by anisomycin, a GST fusion protein containing the first 79 residues of c-Jun (NH_2-Jun^{GST}) was used as a specific JNK substrate (42). Immunocomplexes from untreated or anisomycin-treated CHO^{IR}/PPY^{IRS-1} were prepared with antibodies against JNK1 or IRS-1 and incubated with NH_2-Jun^{GST} and [^{32}P]ATP. Before anisomycin stimulation, αJNK1 and αIRS-1 immunoprecipitates weakly mediated the phosphorylation of NH_2-Jun^{GST}; however, both immunocomplexes prepared from anisomycin-stimulated cells strongly mediated the phosphorylation of NH_2-JNK^{GST} (Fig. 4*F*). The activity of recombinant JNK transiently expressed in 293 cell was tested using ATF-2 as a substrate. Immunoprecipitates of JNK from anisomycin-stimulated 293 transfectants strongly phosphorylated ATF-2, whereas immunoprecipitates of inactive JNK had no effect of ATF-2 phosphorylation (Fig. 4*G*). Similarly, PPY^{IRS-1} was strongly phosphorylated by active but not inactive JNK1 (Fig. 4*G*). Identical results were obtained with IRS-1, which support the hypothesis that JNK1 associated with IRS-1 was activated by anisomycin and mediated phosphorylation of IRS-1.

JNK Phosphorylates Serine 307 of IRS-1—To identify anisomycin-stimulated JNK phosphorylation sites on IRS-1 that might promote the inhibition of insulin-stimulated tyrosine phosphorylation, PPY^{IRS-1} immunoprecipitates from CHO^{IR} cells were incubated without or with recombinant JNK1 prepared in transfected 293 cells (48). PPY^{IRS-1} was used in these experiments to reduce the complexity of the phosphopeptide maps while retaining sensitivity to anisomycin (data not shown). Peptides generated by digestion of phosphorylated PPY^{IRS-1} with trypsin were resolved by reverse-phase HPLC. The HPLC profile of PPY^{IRS-1} phosphorylated with activated JNK contained a single major tryptic phosphopeptide that contained only [^{32}P]phosphoserine (Fig. 5*A*). PPY^{IRS-1} immunoprecipitates incubated with [γ-^{32}P]ATP but without recombinant JNK-1 were much less phosphorylated; however, they contained a phosphopeptide with a similar elution time. The radioactivity in this common peptide was released at Edman cycle 8, suggesting that recombinant JNK and endogenous JNK phosphorylate IRS-1 at a common site. An identical phosphopeptide was also resolved in PPY^{IRS-1} immunoprecipitates from [^{32}P]orthophosphate-labeled CHO^{IR} cells, suggesting that this site is physiologically relevant (Fig. 5*C*).

Based on the deduced amino acid sequence, four tryptic peptides in PPY^{IRS-1} might yield phosphoserine after eight cycles of Edman degradation, including Ser^{272}, Ser^{307}, Ser^{599}, or Ser^{802}; two of these peptides contain a diagnostic endopeptidase Glu-C cleavage site (Fig. 5*D*). To distinguish between these possibilities, the JNK1-stimulated tryptic peak was digested with endoproteinase Glu-C, and a faster eluting peptide was isolated by HPLC (Fig. 5*D*). [^{32}P]Phosphoserine was released from this peptide after six cycles of radiosequencing, which unambiguously revealed Ser^{307} as a major JNK1 phosphorylation site in PPY^{IRS-1} (Fig. 5*D*). This serine residue is conserved in all known IRS-1 homologues and is in a canonical proline-directed serine/threonine kinase phosphorylation motif.

To confirm that Ser^{307} of PPY^{IRS-1} was phosphorylated by JNK1, it was replaced with alanine by site-directed mutagenesis (A307^{PPY}). A307^{PPY} was stably expressed in CHO^{IR} cells and detected by immunoblotting at the predicted molecular size (data not shown). Anisomycin-treated recombinant JNK1 failed to phosphorylate immunoprecipitates of A307^{PPY} incubated with [γ-^{32}P]ATP, confirming that Ser^{307} is a major JNK1 phosphorylation site in IRS-1 (Fig. 6*A*).

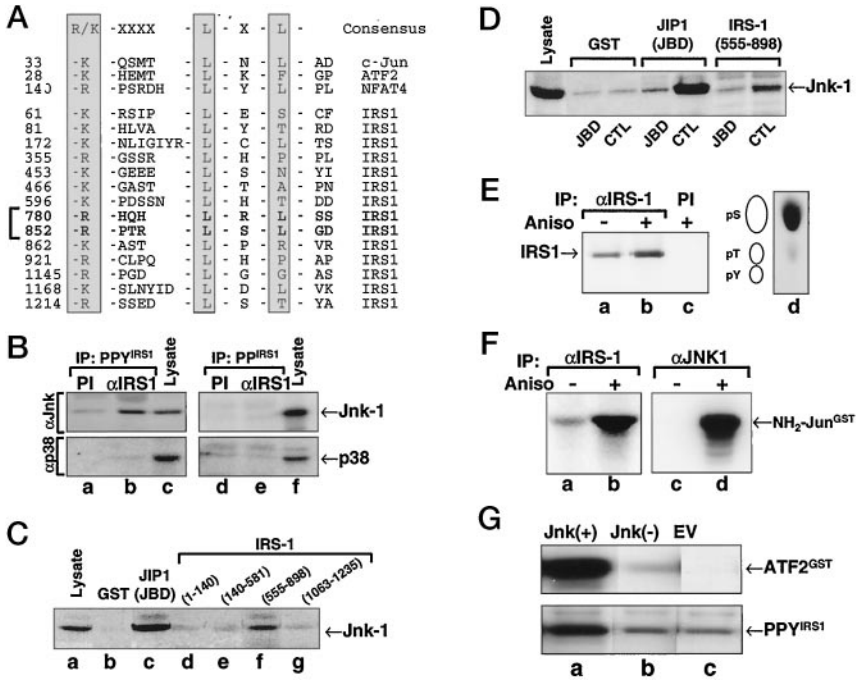

FIG. 4. **JNK1 associates with IRS-1 *in vivo* and *in vitro*.** *A*, schematic diagram depicting JNK-binding domains from c-Jun, ATF2, and NFAT4 for comparison with 14 potential JNK-binding domain (JBDs) found in IRS-1. Those JBDs within PPY^{IRS-1} are indicated (*bracket*). The JBDs most likely to mediate the JNK/IRS-1 interaction are indicated (*boldface type*). *B*, IRS-1 (*lanes b* and *e*) and preimmune (*lanes a* and *d*) immunoprecipitates (*IP*) and cell lysates from CHO^{IR}/PP^{IRS-1} and CHO^{IR}/PPY^{IRS-1} cells were analyzed by immunoblotting with antibodies against JNK1 (*upper panel*) and p38 (*lower panel*). *C*, the JBD of JIP-1 (residues 127–281) and the indicated regions of IRS-1 were expressed as GST fusion proteins (111 pmol) and incubated with 293 cell lysates. Whole cell lysates and GST-pull-downs were analyzed by immunoblotting with antibodies against JNK1. *D*, JIP-1 JBD and IRS-1 amino acids 555–898 GST fusion proteins were incubated in the presence of a 64 μg/ml concentration of either a synthetic peptide designed to the JBD of JIP-1 (residues 148–174) or a control peptide (*CTL*) with cell lysates from 293 cells transfected with JNK1. Whole cell lysates and GST pull-downs were analyzed by immunoblotting with antibodies against JNK-1. *E*, preimmune and IRS-1 IPs from anisomycin-treated CHO^{IR}/PPY^{IRS-1} cells (+) were subjected to an *in vitro* kinase assay (IVK) in the presence of 10 μM LY294002 (*left panel*). Substrates were analyzed by autoradiography after SDS-PAGE and transfer to nitrocellulose. Phosphoamino acid analysis of PPY^{IRS-1} after IVK is shown. *F*, IRS-1 and JNK1 IPs from anisomycin-treated (+) CHO^{IR}/PPY^{IRS-1} cells were subjected to IVK in the presence of 10 μM LY294002 and NH_2-Jun^{GST}. *G*, $ATF2^{GST}$ and PPY^{IRS-1} immunopurified from CHO^{IR}/PPY^{IRS-1} cells were subjected to IVK assay with JNK1 expressed and activated (*lane a*) or not (*lane b*) with 10.0 μg/ml anisomycin in 293 cells. Lysates of 293 cells transfected with empty vector (*EV*) were assayed in parallel (*lane c*).

Ser^{307} Is Critical for the Inhibition of Insulin-stimulated Tyrosine Phosphorylation of IRS-1—CHO^{IR} cells expressing IRS-1 or a $Ser^{307} \rightarrow Ala^{307}$ point mutant ($A307^{IRS-1}$) were treated with anisomycin to determine if Ser^{307} was required for the inhibition of insulin-stimulated tyrosine phosphorylation. Site-directed mutagenesis of Ser^{307} does not alter the structural integrity of IRS-1, since the alanine point mutant stimulates a normal insulin dose response for PI 3-kinase activation (data not shown). As shown above, anisomycin inhibited the insulin-stimulated tyrosine phosphorylation of wild type IRS-1 and reduced its rate of migration during SDS-PAGE before and during insulin stimulation (Fig. 6*B*). By contrast, anisomycin did not reduce the electrophoretic migration of $A307^{IRS-1}$ and had no inhibitory effect on its insulin-stimulated tyrosine phosphorylation (Fig. 6*B*). By comparison, mutation of two potential extracellular signal-regulated kinase phosphorylation sites in IRS-1, $Ser^{612} \rightarrow$ Ala and $Ser^{632} \rightarrow$ Ala, did not prevent the inhibitory effect of anisomycin (data not shown).

Throughout this work, we have used anisomycin to activate JNK in CHO cells. Previous studies showed that TNFα inhibits the insulin-stimulated tyrosine phosphorylation of IRS-1 in myeloid 32D cells (28). To determine whether Ser^{307} is required for the inhibition of IRS-1 tyrosine phosphorylation by TNFα, 32D cells expressing IRS-1 or $A307^{IRS-1}$ were treated with TNFα. As expected, TNFα inhibited the insulin-stimulated tyrosine phosphorylation of wild type IRS-1, whereas it did not inhibit the insulin-stimulated tyrosine phosphorylation of $A307^{IRS-1}$ (Fig. 6*C*). Similar results were also seen in 293 cells in which transfected $A307^{PPY}$ and $A307^{IRS-1}$ did not exhibit decreased insulin-stimulated tyrosine phosphorylation by anisomycin or TNFα in comparison with wild type IRS-1 (data not shown). These results support the hypothesis that the inhibition of insulin-stimulated tyrosine phosphorylation by TNFα and anisomycin requires Ser^{307} of IRS-1 and might be mediated by JNK.

DISCUSSION

Our results show that JNK associates with IRS-1 and phosphorylates IRS-1 mainly at Ser^{307}. This residue is phosphorylated in CHO cells and in IRS-1 immunoprecipitates both before and after the addition of recombinant JNK. Based on several experimental approaches, including the mutation of Ser^{307} to Ala, we conclude that JNK-mediated phosphorylation of Ser^{307} inhibits insulin-stimulated tyrosine phosphorylation of IRS-1. Many of the agents that induce serine/threonine phosphorylation of IRS-1 also activate JNK, including TNFα, interleukin-1α, hyperglycemia, and insulin itself (14, 28, 34, 64–67). Although the phosphorylation of Ser^{307} as a general mechanism to inhibit IRS-1 function is not understood, it might provide a framework to understand the inhibition of insulin or IGF-1 signaling by proinflammatory cytokines under a variety of physiological conditions (23, 24, 28, 29, 34, 68, 69).

The phosphorylation of Ser^{307} is best observed when recombinant IRS-1 and JNK1 are mixed together with [^{32}P]ATP; under these conditions, it is the major site of phosphorylation. Ser^{307} is also phosphorylated in [^{32}P]phosphate-labeled cells and by PI 3-kinase-independent endogenous kinases that associate with IRS-1 during immunoprecipitation. Although we

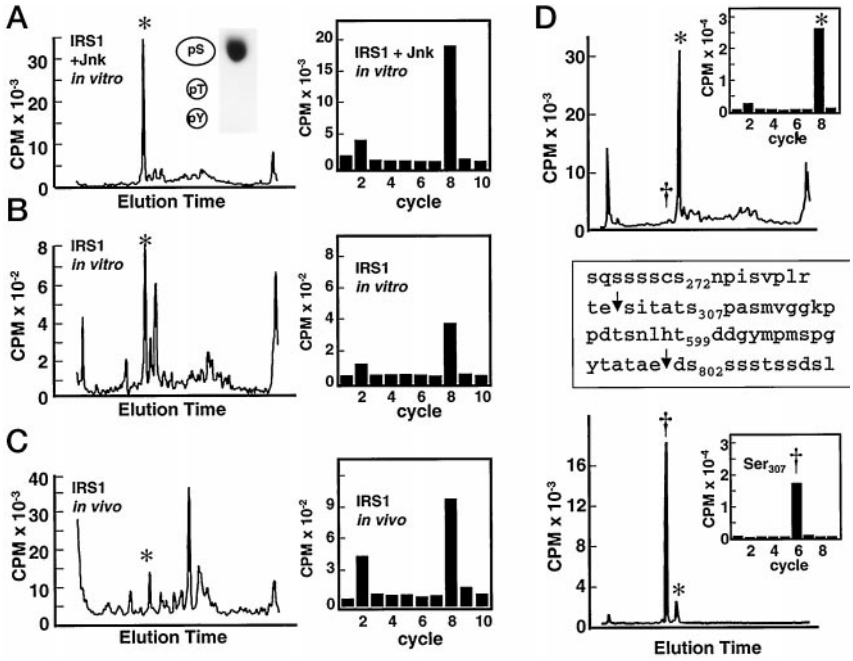

FIG. 5. **Anisomycin-stimulated JNK1 phosphorylates Ser^{307} of PPY^{IRS-1} *in vitro*.** IRS-1 is phosphorylated at a common site *in vivo*, by the associated endogenous kinase, and after IVK with JNK1. Tryptic peptides from PPY^{IRS-1} after immunoprecipitation and phosphorylation with active recombinant JNK1 (*A*), after immunoprecipitation and phosphorylation by the addition of [γ-^{32}P]ATP (*B*), and after immunoprecipitation from ^{32}P-*o*-phosphate-labeled CHO^{IR}/PPY^{IRS-1} cells (*C*) were resolved by reverse phase HPLC. The common phosphopeptide is indicated (*). Phosphoamino acid analysis of the *in vitro* JNK1-catalyzed phosphopeptide is *inset* (*middle panel*). Manual radiosequencing profiles of the common phosphopeptides are shown on the *right*. *D*, HPLC analysis of tryptic peptides of PPY^{IRS-1} after IVK assay with recombinant JNK1 (*upper panel*). HPLC analysis of the tryptic phosphopeptide (*) after digestion with endoproteinase Glu-C is shown in the *lower panel*. The faster eluting Glu-C peak is labeled (†). Manual radiosequencing of the two phosphopeptides after HPLC is *inset*. Four potential tryptic peaks are presented with diagnostic Glu-C cleavage sites denoted (↓). *pS*, phosphoserine; *pT*, phosphothreonine; *pY*, phosphotyrosine.

have no proof that JNK is the endogenous kinase that phosphorylates Ser^{307}, it is the leading candidate. Mutation of Ser^{307} → Ala not only eliminates phosphorylation of IRS-1 by recombinant JNK1 but also abrogates the inhibitory effect of JNK agonists on IRS-1 function. Thus, we conclude that the inhibitory effect of proinflammatory cytokines, like TNFα or interleukin-1β on IRS-1 function might be mediated by JNK.

Insulin and IGF-1 resistance is a common consequence of traumatic injury and chronic obesity. TNFα is produced systemically by macrophages and lymphocytes after inflammatory stimulation or trauma and increases rapidly during experimental injury induced by cerebral ischemic, excitotoxic, and traumatic injury (27). During severe burn injury, TNFα promotes peripheral insulin resistance, whereas it promotes neuronal death in an injured brain, possibly by blocking the ability of IGF-1 to promote repair (21). Adipocytes in obese animals and humans produce TNFα in positive correlation to body mass index and hyperglycemia (23, 24). In adipocytes, TNFα suppresses expression of adipocyte-specific genes and promotes insulin resistance by inhibiting IRS-1 tyrosine phosphorylation (70). Mice lacking TNFα receptors display less insulin resistance during diet-induced obesity (31); blocking TNFα action attenuates ischemic brain damage in rats and mice (22, 25, 26) (32). Thus, TNFα-stimulated serine phosphorylation of IRS proteins might provide a common mechanism for insulin/IGF-1 resistance in a variety of pathological conditions.

TNFα is a multifunctional cytokine that induces a broad spectrum of responses, both at the cellular and organismal level. During TNFα binding, the tumor necrosis factor receptor 1 trimerizes and promotes the assembly of an activated signaling complex, including TRADD, TRAF2, and RIP (37, 38); the germinal center kinase might link this complex to the JNK/p38 protein kinase cascade (37). JNK is activated by at least two MAP kinase kinases (MAPKKs) called MKK4 and MKK7, which are activated by a highly divergent family of MAPK kinase kinase kinases (MAPKKKs) (37, 38, 48, 71, 72). Specificity in this cascade might be established through the assembly of JNK with a specific MAPKK and MAPKKK at a common scaffold protein. Recently, the JIP was shown to specifically associate with MEKK1, MKK7, and JNK, which mediate JNK activation (48). Moreover, JNK is known to associate with many of its substrates, which provides additional levels of signaling specificity.

JNK associates with IRS-1 in CHO cells and co-purifies with IRS-1 during immunoprecipitation. The mechanism that mediates this association is unknown, but the association might occur through a direct interaction of JNK at putative JNK binding motifs in the COOH terminus of IRS-1. IRS-1 contains several putative JNK binding motifs. The region of IRS-1 (amino acids 555–898) responsible for JNK1 association *in vitro* contains two amino acid sequence motifs that are similar to the JNK binding motifs in the JIPs (56). Mutation of the conserved leucine residues in these motifs significantly reduces the binding of JNK.[2] However, truncated IRS-1 molecules lacking these motifs retain sensitivity to anisomycin, suggesting that other binding sites might be utilized in cells.

The assembly of multicomponent complexes appears to be essential for regulation of the JNK signaling system, since it promotes efficient and specific activation of JNK with upstream and downstream elements (48). Thus, the interaction between IRS-1 and JNK is likely to be critical for the phosphorylation of Ser^{307}. The apparent specificity of JNK for one residue is remarkable, given the presence of multiple Ser-Pro motifs in IRS-1. Perhaps the orientation imposed by the interaction between JNK and IRS-1 imparts regiospecificity on the

[2] V. Aguirre, T. Uchida, L. Yenush, R. Davis, and M. F. White, unpublished results.

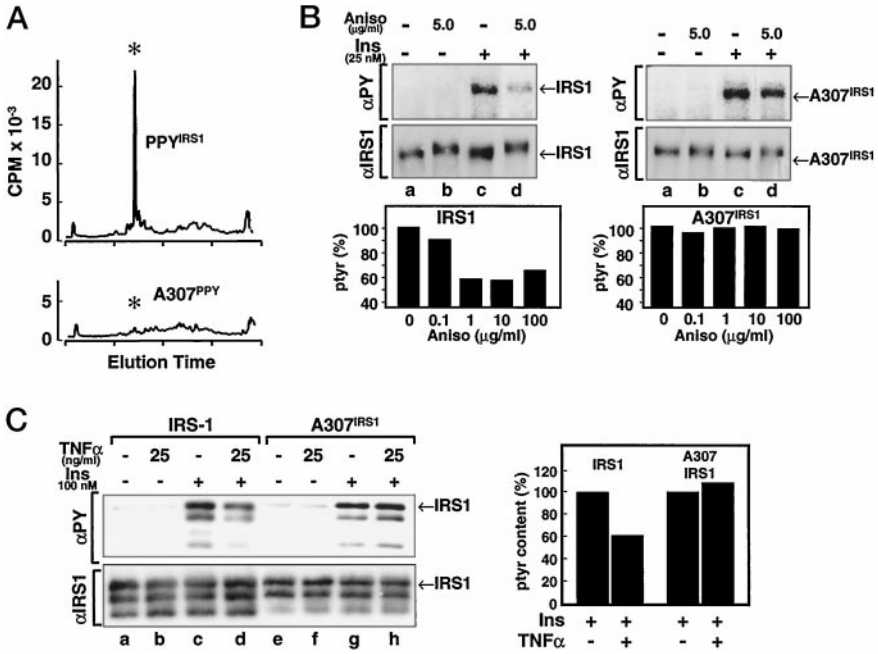

FIG. 6. **Anisomycin and TNFα require Ser^{307} of IRS-1 for the inhibition of insulin signaling.** *A*, a Ser^{307} → Ala^{307} PPY^{IRS-1} (A307^{PPY}) point mutant is not phosphorylated by JNK1 *in vitro.* HPLC analysis of tryptic peptides generated from PPY^{IRS-1} and A307^{PPY} phosphorylated with recombinant JNK1 is shown. *B*, CHO/IRS-1 and CHO/A307^{IRS-1} (a Ser^{307} to Ala^{307} point mutant in full-length IRS-1) cells were treated for 30 min with the indicated doses of anisomycin (*Aniso*) prior to stimulation with insulin (*Ins*). Lysates were analyzed by Western blot using antibodies against phosphotyrosine and IRS-1. The anisomycin dose response (*bottom panels*) was quantified as phosphotyrosine content of IRS-1 per unit of protein. Similar results were obtained with three mutant cell line clones. *C*, 32D/IRS-1 and 32D/A307^{IRS-1} cells were treated for 4 h with (*lanes b*, *d*, *f*, and *h*) or without (*lanes a*, *c*, *e*, and *g*) 25 ng/ml TNFα prior to stimulation with insulin (*lanes c*, *d*, *g*, and *h*). Lysates were analyzed by Western blot using antibodies against phosphotyrosine and IRS-1. Data presented at the *right* is quantified as phosphotyrosine content of IRS-1 per unit of protein. Similar results were obtained with three mutant cell line clones. Additional bands of higher electrophoretic mobility than full-length IRS-1 in the immunoblots of 32D/IRS-1 cell lysates represent degradation products (33).

phosphorylation reaction. Since JNK associated with IRS-1 is activated during stimulation of CHO cells with anisomycin, there must be a mechanism for interaction between JNK and relevant upstream MAPKKs and MAPKKKs while JNK is associated with IRS-1. One possibility is that a MAPKKK and MAPKK bind directly to IRS-1. Alternatively, a JNK scaffold protein, like JIP, might associate directly with IRS-1 to bring JNK and its regulatory elements to IRS-1. The COOH terminus of JIP contains a putative PTB domain and Src homology 3 domain, which might mediate association with IRS-1 (48).

Insulin itself stimulates serine phosphorylation of IRS-1. At least part of this response might depend on protein kinase B, which is activated through the PI 3-kinase cascade (73). Although protein kinase B does not appear to directly phosphorylate IRS-1, downstream kinases activated by protein kinase B promote serine phosphorylation of IRS-1 that enhances subsequent tyrosine phosphorylation. Insulin-stimulated serine/threonine kinases may also promote a negative feedback on IRS-1 tyrosine phosphorylation. Previous reports suggest that JNK is activated by insulin (51, 74). Furthermore, JNK is activated in a PI 3-kinase-dependent manner in some cell systems (63). Thus, JNK might promote feedback inhibition of IRS-1 tyrosine phosphorylation through phosphorylation of Ser^{307}.

The mechanism by which the phosphorylation of Ser^{307} inhibits IRS-1 tyrosine phosphorylation is not known. TNFα, calyculin A, 12-*O*-tetradecanoylphorbol-13-acetate, and prolonged insulin stimulation inhibit IRS-1 and IRS-2 binding to the phosphorylated NPEY motif in the juxtamembrane region of the insulin receptor β-subunit (69). Moreover, we found that the inhibitory effects of anisomycin and TNFα are lost upon deletion of the PTB domain in IRS-1. Since Ser^{307} is adjacent to the PTB domain, its phosphorylation might disrupt the interaction between the phosphorylated NPEY motif of the insulin receptor and IRS-1. Although the PTB domain is not absolutely required for IRS-1 tyrosine phosphorylation, its presence strongly facilitates the process, and partial disruption of PTB domain function is expected to reduce coupling of the insulin receptor to IRS-1 at endogenous levels of these proteins (61, 62). Alternatively, phosphorylation of Ser^{307} might recruit signaling molecules, which sterically inhibit interactions between the PTB domain and the insulin receptor β-subunit. Future experiments will reveal the mechanism by which Ser^{307} phosphorylation inhibits IRS-1 tyrosine phosphorylation.

The phosphorylation of serine residues in the COOH terminus of IRS-1 also regulates insulin-stimulated tyrosine phosphorylation. Four serine residues in the COOH-terminal tail of IRS-1, Ser^{612}, Ser^{632}, Ser^{662}, and Ser^{731}, are present in YMXMSP motifs, adjacent to insulin-stimulated tyrosine phosphorylation sites (20, 57, 60, 66). During insulin stimulation, the tyrosine residues are phosphorylated and bind to the Src homology 2 domains of p85 to activate PI 3-kinase (7). Inhibition of IRS-1 tyrosine phosphorylation by phorbol 12-myristate 13-acetate or endothelin-1 requires Ser^{612}, whereas inhibition by platelet-derived growth factor depends on Ser^{632}, Ser^{662}, and Ser^{731} (14). Phosphorylation of these residues might block the local phosphorylation at the adjacent tyrosine, providing a limited and specific inhibitory effect. By contrast, these residues do not mediate the inhibitory effect of okadaic acid (14, 57, 66), anisomycin, or TNFα.[2]

In summary, many serine residues may negatively regulate IRS-1 tyrosine phosphorylation. Phosphorylation of Ser^{307} is critical for the inhibitory effect of anisomycin and TNFα, which might be mediated by the association of activated JNK with IRS-1. Our results are consistent with the hypothesis that phospho-Ser^{307} blocks the interaction between the IRS-1 PTB domain and the insulin receptor, which might significantly reduce the coupling between the activated insulin receptor and

IRS-1. The phosphorylation state of Ser^{307} might predict, in certain cells or tissues, the ability of IRS-1 to mediate the insulin response. Thus, detection and reversal of Ser^{307} phosphorylation might be a powerful tool for the pharmaceutical intervention of the progression of insulin resistance and type II diabetes.

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