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c-Jun N-terminal Kinase (JNK) Mediates Feedback Inhibition of the Insulin Signaling Cascade*

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Yong Hee Lee‡§, Jodel Giraud‡, Roger J. Davis¶‖, and Morris F. White‡‖**

From the ‡Howard Hughes Medical Institute, Joslin Diabetes Center, Harvard Medical School, Boston, Massachusetts 02215 and ¶Howard Hughes Medical Institute, Program in Molecular Medicine, Department of Biochemistry and Molecular Biology, University of Massachusetts Medical School, Worcester, Massachusetts 01605

Activation of the c-Jun N-terminal kinase (JNK) by proinflammatory cytokines inhibits insulin signaling, at least in part, by stimulating phosphorylation of rat/mouse insulin receptor substrate 1 (Irs1) at Ser^{307} (Ser^{312} in human IRS1). Here we show that JNK mediated feedback inhibition of the insulin signal in mouse embryo fibroblasts, 3T3-L1 adipocytes, and $32D^{IR}$ cells. Insulin stimulation of JNK activity required phosphatidylinositol 3-kinase and Grb2 signaling. Moreover, activation of JNK by insulin was inhibited by a cell-permeable peptide that disrupted the interaction of JNK with cellular proteins. However, the direct binding of JNK to Irs1 was not required for its activation by insulin, whereas direct binding was required for Ser^{307} phosphorylation of Irs1. Insulin-stimulated Ser^{307} phosphorylation was reduced 80% in cells lacking JNK1 and JNK2 or in cells expressing a mutant Irs1 protein lacking the JNK binding site. Reduced Ser^{307} phosphorylation was directly related to increased insulin-stimulated tyrosine phosphorylation, Akt phosphorylation, and glucose uptake. These results support the hypothesis that JNK is a negative feedback regulator of insulin action by phosphorylating Ser^{307} in Irs1.

Insulin resistance is a common problem that is associated with obesity and hypertension, infection and injury, and type 2 diabetes, in which β-cells fail to secrete sufficient insulin to compensate for peripheral insulin resistance (1–3). Insulin signaling complexes are assembled by insulin-stimulated tyrosine phosphorylation of scaffold proteins, including the Irs1 [1] proteins, Shc, APS and Shc, Gab1/2, Dock1/2 and cbl (4–6). Although the role of each of these substrates merits attention, work with transgenic mice reveals the importance of Irs1 and Irs2 for somatic growth and carbohydrate metabolism (7, 8). Tyrosine phosphorylation sites in Irs1 and Irs2 bind to the Src homology-2 domain in various signaling proteins that mediate the insulin response, including PI 3-kinase, Grb2, Shp2, Crk, and others (9).

A number of mechanisms might contribute to the dysregulation of the insulin-signaling pathway, including serine phosphorylation of the insulin receptor or the Irs proteins, degradation of Irs proteins, or altered activity of phosphoprotein or phospholipid phosphatases (10–16). Irs1 and Irs2 contain many potential serine or threonine phosphorylation sites that might play regulatory roles during the insulin response. Various metabolites associated with insulin resistance stimulate serine phosphorylation of Irs1, including free fatty acids, diacylglycerol, fatty acyl CoAs, ceramides, and glucose (17). Moreover, proinflammatory cytokines, especially interferon γ, interleukin 1β, or TNFα produced during infection, injury, or chronic obesity stimulate serine phosphorylation of Irs1 and cause insulin resistance (18, 19). Insulin itself induces serine phosphorylation of Irs1, suggesting that chronic compensatory hyperinsulinemia in response to stress-induced insulin resistance might exacerbate the problem (20).

More than 100 potential serine phosphorylation sites exist in Irs1, and many protein kinases phosphorylate Irs1, including JNK, protein kinase Cζ, IKKβ, mammalian target of rapamycin, MAPK, and AMPK (11–13, 21–23); however, the kinases and phosphorylation sites that are physiologically important for Irs1 function are difficult to resolve. Although many kinases phosphorylate Irs1, JNK is especially interesting because it associates with Irs1 and phosphorylates Ser^{307} (11, 20). Insulin also stimulates Ser^{307} phosphorylation in various cultured cell lines; and the orthologous site in human Irs1 (Ser^{312}) is phosphorylated in muscle during a hyperinsulinemic clamp (24). Ser^{307} is located next to the phosphotyrosine-binding domain in Irs1, and its phosphorylation inhibits the interaction of the phosphotyrosine-binding domain with the phosphorylated NPEY motif in the activated insulin receptor (20). Because the phosphotyrosine-binding domain is important for efficient insulin-stimulated tyrosine phosphorylation of Irs1, Ser^{307} phosphorylation might contribute to insulin resistance during physiological stress.

JNK is a member of the MAP kinase family of protein kinases, which also includes ERK and p38 (25, 26). JNK is activated by proinflammatory cytokines induced during microbial infection or thermal or mechanical injury (27, 28). Three JNK isoforms, JNK1, JNK2, and JNK3, are expressed in multiple splice variants (29). Disruption of these genes in mice reveals that JNK1 and JNK2 mediate T cell activation and brain development (30–32), and JNK3 mediates neuronal apo-

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‖ Investigators of the Howard Hughes Medical Institute.

** To whom correspondence should be addressed: Howard Hughes Medical Institute, Joslin Diabetes Center, 1 Joslin Place, Boston, MA 02215. Tel.: 617-732-2578; Fax: 617-732-2593; E-mail: morris.white@joslin.harvard.edu.

[1] The abbreviations used are: Irs, insulin receptor substrate; JNK, c-Jun N-terminal kinase; PI, phosphatidylinositol; TNF, tumor necrosis factor; TNFR1, tumor necrosis factor receptor 1; IKK, IκB kinase-β; ERK, extracellular signal-regulated kinase; FBS, fetal bovine serum; DMEM, Dulbecco's modified Eagle's medium; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; MEF, mouse embryo fibroblast; JBP, JNK-binding peptide; Jip, JNK-interacting protein; HIV, human immunodeficiency virus; MAPK, mitogen-activated protein kinase; AMPK, AMP-activated kinase.

ptosis in the hippocampus (33). Various growth factors also activate JNK, including prolactin, epidermal growth factor, nerve growth factor and platelet-derived growth factor, insulin-like growth factor 1, and ligands for some G protein-coupled receptors (34–40). Insulin stimulates JNK in various cells and tissues, but the mechanisms involved and the role of JNK during insulin action are poorly defined (41–43). Here we show that insulin-stimulated JNK associates with Irs1 and phosphorylates Ser^{307}, which inhibits insulin signaling. Thus, JNK might serve a dual function as a heterologous inhibitor of insulin action during acute and chronic inflammation and as a feedback inhibitor during insulin stimulation.

MATERIALS AND METHODS

Antibodies and Reagents—Antibodies against JNK, phospho-JNK ($pThr^{183}/pTyr^{185}$), Pkb/Akt, and phospho-Pkb/Akt ($pSer^{473}$) were purchased from New England Biolabs. Monoclonal antibodies against JNK and phospho-c-Jun ($pSer^{63}$) were purchased from Santa Cruz. Phosphotyrosine antibody (PY20) was purchased from Transduction Laboratories. Antibodies against Irs1 and phosphorylated Ser^{307} in Irs1 were described previously (20, 24). Insulin was purchased from Roche Molecular Biochemicals, and TNFα was purchased from R&D Systems. LY294002 and PD98059 were purchased from Calbiochem-Novabiochem Corp. Peptides were synthesized by Boston Biomolecules and purified by high pressure liquid chromatography, and the sequences were confirmed by mass spectrometry.

Cell Culture—Murine myeloid progenitor 32D cells were maintained in RPMI 1640 medium supplemented with 10% FBS, 5% WEHI conditioned medium (as a source of interleukin-3), and 5 mM histidinol and made quiescent by serum starvation for 4 h (20). 32D transfectants were generated by electroporation and selected in histidinol as described previously (44); site-directed mutagenesis of the JNK binding motif in *Irs1* was described previously (20). Mouse embryo fibroblasts from wild type or *JNK1*::*JNK2* knockout mice were grown in DMEM with 10% FBS (45). 3T3-L1 preadipocytes were maintained at 37 °C in 10% CO_2 in DMEM containing 2 mM glucose and 10% calf serum. These cells were differentiated into adipocytes by incubation for 3 days in DMEM supplemented with 25 mM glucose, 1 μM insulin, 0.5 mM 3-isobutylmethylxanthine, 1 μM dexamethasone, and 10% FBS and 3 days in DMEM supplemented with 1 μM insulin and 10% FBS (46). More than 90% differentiation was achieved after 4–9 days in DMEM containing 25 mM glucose and 10% FBS with no other additives.

Cell Lysis, Immunoprecipitation, and Western Blot Analysis—Cells were lysed in 20 mM Tris (pH 7.4) containing 150 mM NaCl, 1% Nonidet P-40, 5 mM EDTA, 10 mM NaF, 10 mM pyrophosphate, 100 μM $NaVO_4$, 1 mM phenylmethanesulfonyl fluoride, 5 μg/ml leupeptin, and 5 μg/ml protinin. Lysates were resolved by SDS-PAGE, transferred to nitrocellulose, and proteins were detected by immunoblotting and chemiluminescence (Amersham Biosciences). To analyze the association of Irs1 with JNK in 32D cells, immunoprecipitation was performed with Irs1 antibody immobilized on protein G-Sepharose using a Seize™ X protein G immunoprecipitation kit (Pierce) and analyzed by immunoblotting with monoclonal JNK antibody. For 3T3-L1 adipocytes, immunoprecipitates with monoclonal JNK antibody were analyzed by immunoblotting with Irs1 antibody.

2-Deoxyglucose Uptake in 3T3-L1 Adipocytes—Fully differentiated 3T3-L1 adipocytes were placed in DMEM containing 5 mM glucose and 0.1% bovine serum albumin for 2 h at 37 °C. Before glucose transport measurements, cells were washed with KRH buffer (20 mM HEPES (pH 7.4) 1.25 mM $MgSO_4$, 1.25 mM $CaCl_2$, 136 mM NaCl, 4.7 mM KCl, and 0.1% bovine serum albumin) and incubated with synthetic peptides before insulin stimulation. Glucose transport was determined by the addition of 0.1 mM 2-deoxyglucose containing 0.5 μCi of 2-[1,2-^{3}H]-deoxy-D-glucose (PerkinElmer Life Sciences) as described previously (47). Nonspecific uptake was assessed in the presence of 10 μM cytochalasin B and subtracted from all of the measured values. Glucose transport experiments were terminated after 10 min by aspiration by four washes with ice-cold phosphate-buffered saline. Cells were lysed in 0.1% SDS in phosphate-buffered saline, and radioactivity was determined by scintillation counting.

RESULTS

Irs1 Is Necessary for the Insulin-induced Activation of JNK—We used 32D cell lines stably expressing the human insulin receptor alone or with rat Irs1 to determine the function of insulin-stimulated JNK. 32D cells are murine myeloid progenitors that express few endogenous insulin receptors and no Irs proteins and require interleukin-3 for growth (48); however, they naturally express JNK1 and JNK2 (data not shown). JNK1 and JNK2 are activated by tandem Thr/Tyr phosphorylation (Thr^{183} and Tyr^{185} in JNK1), which is detected in both isoforms by immunoblotting with a phosphospecific-JNK antibody (αJNK^{PI}). Both JNK homologs are variably expressed as two alternative transcripts that yield a 46-kDa and a 54-kDa isoform that are detected by αJNK^{PI}. In this report we do not distinguish between the homologs.

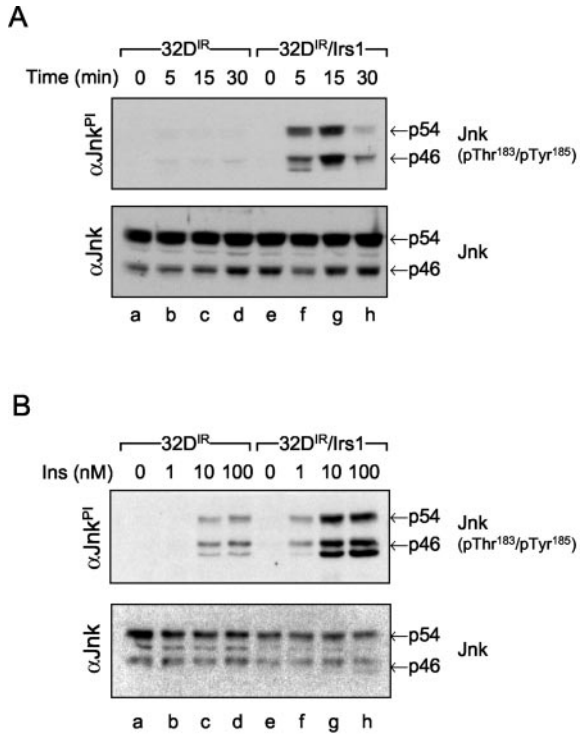


FIG. 1. **Irs1 is necessary for the insulin-induced activation of JNK in 32D cells.** *A*, total cell lysates from $32D^{IR}$ cells stably transfected with empty vector or Irs1-expressing vector treated with 10 nM insulin for the indicated times were analyzed by immunoblotting with anti-phospho-JNK (αJnk^{PI}) and anti-JNK (αJnk) antibodies. *B*, total cell lysates from $32D^{IR}$ cells stably transfected with empty vector or Irs1-expressing vector treated with the indicated doses of insulin for 15 min were analyzed by immunoblotting with anti-phospho-JNK and anti-JNK antibodies.

Before insulin stimulation, JNK phosphorylation was not detected by αJNK^{PI}, suggesting that under basal conditions JNK was not activated. However, the 46- and 54-kDa isoforms of JNK were phosphorylated 5 min after insulin was added to the $32D^{IR}$/Irs1 cells (Fig. 1*A*). JNK phosphorylation was maximal at 10 nM insulin in $32D^{IR}$/Irs1 cells, whereas it was weakly phosphorylated during insulin stimulation of $32D^{IR}$ cells (Fig. 1*B*). Thus, Irs1 was required in $32D^{IR}$ cells for maximal sensitivity of JNK to insulin stimulation.

JNK Promotes Ser^{307} Phosphorylation of Irs1 During Insulin Stimulation—We investigated the relationship between insulin-stimulated JNK activity and Ser^{307} phosphorylation of rat Irs1 in transfected $32D^{IR}$/Irs1 cells. During insulin stimulation, tyrosine-phosphorylated Irs1 activates the PI 3-kinase → Pkb/Akt cascade that can be inhibited by LY294002 and activates the Grb2/Sos/Ras → ERK1/2 cascade that can be inhibited by PD98059 (9). Each compound inhibited insulin-stimulated phosphorylation of the 46- and 54-kDa isoforms of JNK in $32D^{IR}$/Irs1 cells and inhibited JNK-mediated phosphorylation of c-Jun (Fig. 2*A*). Thus, PI 3-kinase and MEK1 mediated JNK activation in insulin-stimulated $32D^{IR}$/Irs1 cells (Fig. 2*A*). Each

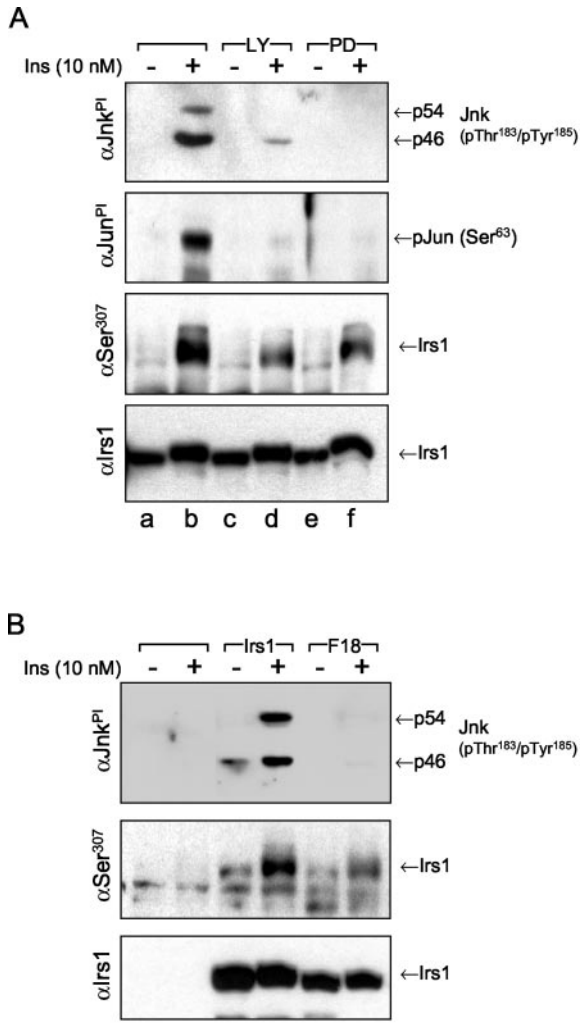


FIG. 2. **Insulin induces the activation of JNK and Ser^{307} phosphorylation of Irs1 in 32D cells.** *A*, total cell lysates from 32D^{IR} cells stably expressing Irs1 treated with LY294002 or PD98059 for 30 min before 15 min of stimulation with 10 nM insulin (*Ins*) were analyzed by immunoblotting with anti-phospho-JNK (αJnk^{PI}), anti-phospho-c-Jun (αJun^{PI}), anti-phospho-Ser^{307} (αSer^{307}), and anti-Irs1 (α*Irs1*) antibodies. *B*, total cell lysates from 32D^{IR} cells stably expressing wild type Irs1 or F18 mutant Irs1 in which all 18 tyrosine residues were mutated to phenylalanine, treated with 10 nM insulin (*Ins*) for 15 min were analyzed by immunoblotting with anti-phospho-JNK, anti-phospho-Ser^{307}, and anti-Irs1 antibodies.

drug also inhibited by 75% the insulin-stimulated phosphorylation of Ser^{307} in Irs1, suggesting that the majority of Ser^{307} phosphorylation might be mediated by JNK. However, other insulin-stimulated kinases appear to be involved (Fig. 2*A*).

To confirm that tyrosine phosphorylation of Irs1 was required for activation of JNK by insulin, 32D^{IR} cells were transfected with F18^{Irs1}, a rat Irs1 mutant that lacks all the known tyrosine phosphorylation sites, including those that activate the PI 3-kinase and ERK pathways (48). Consistent with the inhibitory effect of LY294002 or PD98059 on JNK phosphorylation, insulin failed to stimulate JNK phosphorylation in 32D^{IR}/F18^{Irs1} cells (Fig. 2*B*). Insulin-stimulated phosphorylation of Ser^{307} was also significantly reduced. However, like inhibition by LY294002 or PD98059, a minor pathway independent of Irs1 tyrosine phosphorylation might be involved (Fig. 2*B*).

Insulin-stimulated Ser^{307} Phosphorylation of Irs1 Is Reduced in Mouse Embryo Fibroblasts (MEFs) Lacking JNK1 and JNK2—To further establish the role of JNK in insulin signaling, MEFs lacking both JNK1 and JNK2 (*JNK1*$^{-/-}$::*JNK2*$^{-/-}$

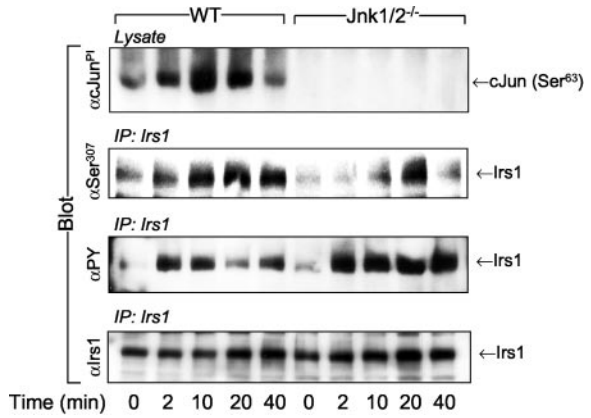
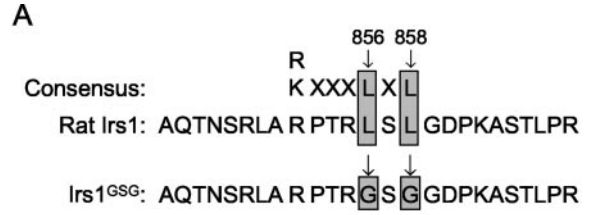


FIG. 3. **Insulin-stimulated Ser^{307} phosphorylation of Irs1 is reduced in mouse embryo fibroblasts lacking JNK1 and JNK2.** Wild type (*WT*) or JNK1 and JNK2 double knock-out (*Jnk1/2*$^{-/-}$) MEF cells were deprived of serum for 4 h before stimulation with insulin. Total cell lysates or immunoprecipitates (*IP*) of Irs1 from MEF cells treated with 10 nM insulin for the indicated times were analyzed by immunoblotting with anti-phospho-Ser^{307} (α*Ser^{307}*), anti-phosphotyrosine (α*PY*), anti-Irs1 (α*Irs1*), and anti-phospho-c-Jun (αJun^{PI}) antibodies.



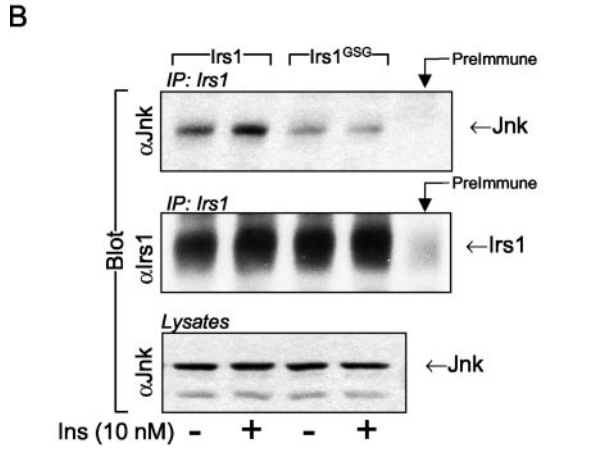


FIG. 4. **Mutation at JNK-binding motif of Irs1 abrogates the interaction between Irs1 and JNK in 32D cells.** *A*, a schematic of the JNK-binding motif in the rat Irs1 sequence and the pair of leucine residues at positions 856 and 858 that were mutated to glycine residues. *B*, immunoprecipitates (*IP*) of Irs1 or total cell lysates from 32D^{IR} cells stably expressing wild type Irs1 or mutant Irs1^{GSG} treated with 10 nM insulin for 10 min were analyzed by immunoblotting with monoclonal anti-JNK (α*Jnk*) and anti-Irs1 (α*Irs1*) antibodies.

MEFs) were used (45). Insulin-stimulated JNK activity assayed by immunoblotting c-Jun phosphorylation was completely absent in *JNK1*$^{-/-}$::*JNK2*$^{-/-}$ MEFs, whereas c-Jun phosphorylation was stimulated maximally in wild type cells 10 min after insulin stimulation (Fig. 3). Insulin induced Ser^{307} phosphorylation of Irs1 in wild type MEFs, whereas phosphorylation was significantly reduced in *JNK1*$^{-/-}$::*JNK2*$^{-/-}$

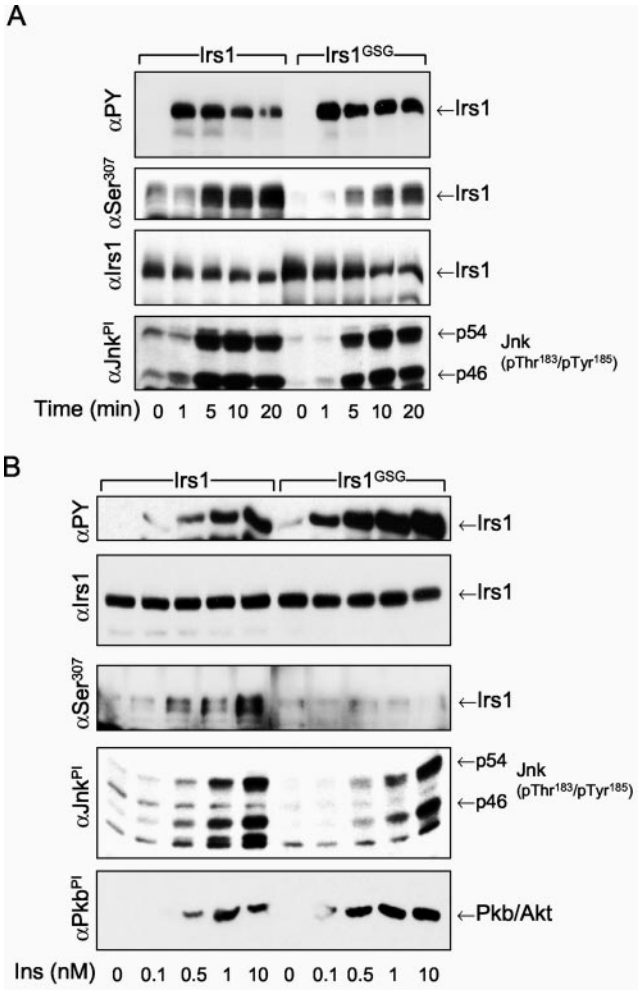


FIG. 5. **Mutation at JNK-binding motif in Irs1 reduces Ser^{307} phosphorylation and enhances Irs1 tyrosine phosphorylation in 32D cells.** *A*, total cell lysates from 32D^{IR} cells stably expressing wild type Irs1 or mutant Irs1^{GSG} treated with 10 nM insulin for the indicated times were analyzed by immunoblotting with anti-phosphotyrosine (α*PY*), anti-phospho-Ser^{307} (α*Ser^{307}*), anti-Irs1 (α*Irs1*), and anti-phospho-JNK (α*Jnk^{PI}*) antibodies. *B*, total cell lysates from 32D^{IR} cells stably expressing wild type Irs1 or mutant Irs1^{GSG} treated with the indicated doses of insulin for 10 min were analyzed by immunoblotting with anti-phosphotyrosine (α*PY*), anti-phospho-Ser^{307} (α*Ser^{307}*), anti-Irs1, anti-phospho-JNK (α*Jnk^{PI}*), and anti-phospho-Pkb/Akt (α*Pkb^{PI}*) antibodies.

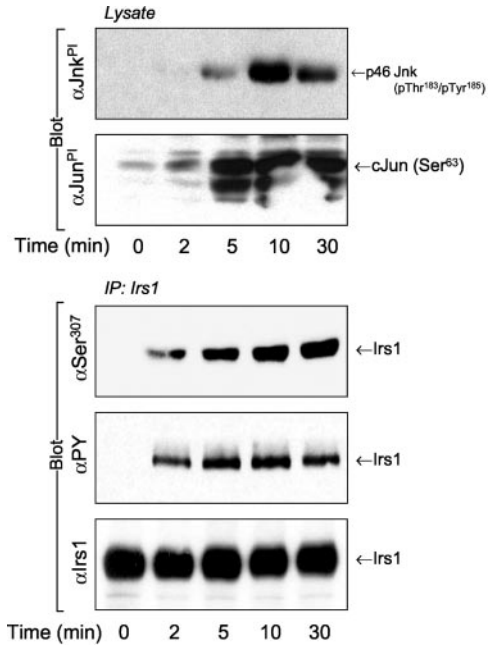


FIG. 6. **Insulin induces the activation of JNK and Ser^{307} phosphorylation of Irs1 in 3T3-L1 adipocytes.** Fully differentiated 3T3-L1 adipocytes were deprived of serum overnight. Total cell lysates or immunoprecipitates (*IP*) of Irs1 from 3T3-L1 adipocytes treated with 10 nM insulin for the indicated times were analyzed by immunoblotting with anti-phospho-JNK (α*Jnk^{PI}*), anti-phospho-c-Jun (α*Jun^{PI}*), anti-phospho-Ser^{307} (α*Ser^{307}*), anti-phospho-tyrosine (α*PY*) and anti-Irs1 (α*Irs1*) antibodies.

MEFs. However, transient Ser^{307} phosphorylation was detected reproducibly after 20 min of insulin stimulation and might represent the JNK-independent pathway (Fig. 3). Insulin-stimulated tyrosine phosphorylation of Irs1 was increased in the *JNK1$^{-/-}$*::*JNK2$^{-/-}$* MEFs. Tyrosine phosphorylation of Irs1 ordinarily declined after 20 min of insulin stimulation in wild type cells; however, it remained high for at least 40 min in *JNK1$^{-/-}$*::*JNK2$^{-/-}$* MEFs. Thus, JNK is a major kinase responsible for insulin-induced Irs1 Ser^{307} phosphorylation that inhibits insulin-stimulated tyrosine phosphorylation.

The Role of the JNK-binding Motif in Irs1—JNK binds specifically to scaffold proteins that mediate its interaction with upstream regulatory kinases and downstream substrates. The consensus amino acid sequence motif that binds to JNK (JNK-binding peptide (JBP)) is best characterized by alignment of JNK-interacting protein 1 (Jip1) and Jip2 (49). Previous studies reveal that two leucine residues within the JNK-binding motif are essential for JNK binding (49). Irs1 contains a similar JNK-binding motif, including both leucine residues at positions 856 and 858 (20). We prepared 32D^{IR} cells expressing mutant Irs1 proteins that contain glycine substitutions for the leucine residues (Irs1^{GSG}) to determine the effect of blocking JNK binding to Irs1 on the activation of JNK and ability to phosphorylate Ser^{307} (Fig. 4*A*). Insulin stimulated the binding of wild type Irs1 to JNK in 32D^{IR}/Irs1 cells, whereas insulin failed to stimulate this interaction between JNK and Irs1^{GSG}, confirming that the JNK-binding motif was required (Fig. 4*B*).

During insulin stimulation, wild type Irs1 and Irs1^{GSG} were rapidly tyrosine phosphorylated, but phosphorylation of Irs1 rapidly declined, whereas that of Irs1^{GSG} was sustained for at least 20 min (Fig. 5*A*). Although both Irs1 and Irs1^{GSG} mediated insulin-stimulated JNK activation, only Irs1 was susceptible to inhibition by Ser^{307} phosphorylation (Fig. 5*A*). These results reveal that direct binding of JNK to Irs1 was not required for insulin stimulation, whereas direct binding was required for JNK-mediated Ser^{307} phosphorylation. Moreover, Irs1^{GSG} displayed more sensitive and intense tyrosine phosphorylation than wild type Irs1 at every concentration of insulin tested (Fig. 5*B*). Consistent with increased tyrosine phosphorylation, Irs1^{GSG} mediated more phosphorylation of Pkb/Akt during insulin stimulation (Fig. 5*B*). By contrast, the time course of JNK phosphorylation was not significantly affected by the Irs1^{GSG} mutant, whereas the sensitivity of JNK phosphorylation to insulin might be slightly impaired (Fig. 5*B*). These results support the hypothesis that an interaction between JNK and Irs1 was not essential for activation but was required for insulin-stimulated Ser^{307} phosphorylation.

Cell-permeable JNK-binding Motif Inhibits Activation of JNK by Insulin—Insulin stimulated phosphorylation of the 46-kDa JNK isoform in fully differentiated 3T3-L1 adipocytes, which increased its activity as revealed by phosphorylation of c-Jun (Fig. 6). Consistent with previous results, insulin strongly stimulated Ser^{307} phosphorylation of Irs1 in these cells; Irs1 was also tyrosine-phosphorylated during insulin stimulation (Fig. 6). The effect of Ser^{307} phosphorylation on insulin signaling in 3T3-L1 adipocytes was investigated by

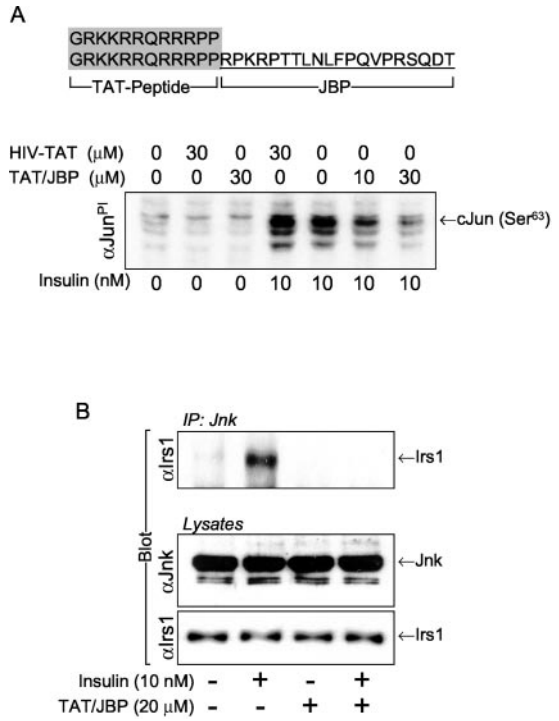


FIG. 7. **Cell-permeable peptide inhibitor of JNK inhibits the interaction between Irs1 and JNK in 3T3-L1 adipocytes.** *A*, total cell lysates from 3T3-L1 adipocytes treated with control (*TAT-Peptide*) or TAT/JBP at the indicated concentrations for 30 min before 10 min of stimulation with 10 nM insulin were analyzed with anti-phospho-c-Jun (αJun^{PI}) antibody. *B*, immunoprecipitates (*IP*) of JNK using monoclonal anti-JNK antibody or total cell lysates from 3T3-L1 adipocytes treated with or without 20 μM TAT/JBP for 30 min before 10 min of stimulation with 10 nM insulin were analyzed by immunoblotting with anti-Irs1 ($\alpha Irs1$) and monoclonal anti-JNK (αJnk) antibodies.

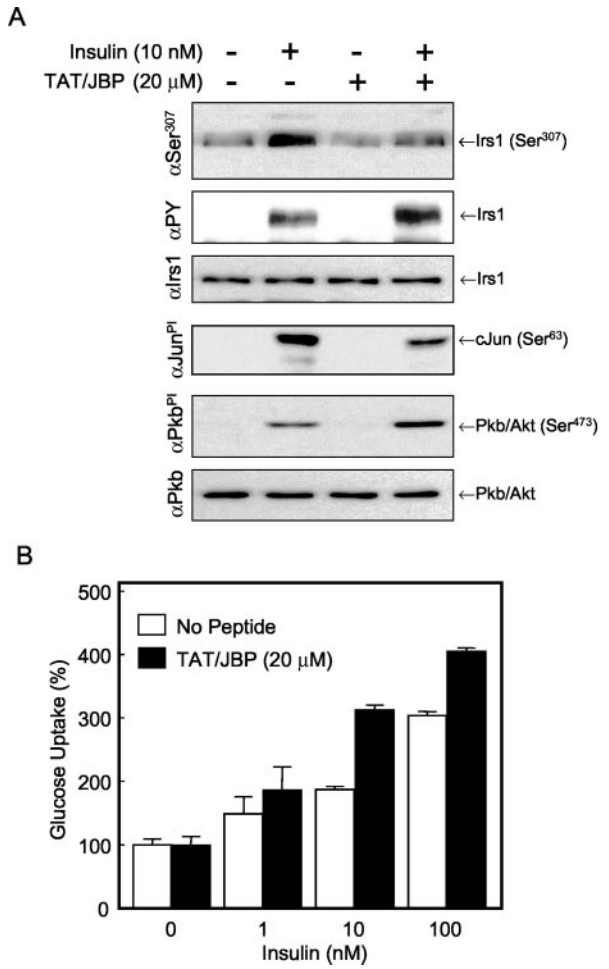


FIG. 8. **Inhibition of JNK activity reduces Ser^{307} phosphorylation of Irs1 and enhances insulin signal transduction and glucose uptake in 3T3-L1 adipocytes.** *A*, total cell lysates from 3T3-L1 adipocytes treated with or without 20 μM TAT/JBP for 30 min before 10 min of stimulation with 10 nM insulin were analyzed by immunoblotting with anti-phospho-Ser^{307} (αSer^{307}), anti-phosphotyrosine (αPY), anti-Irs1 ($\alpha Irs1$), anti-phospho-c-Jun (αJun^{PI}), anti-phospho-Pkb/Akt (αPkb^{PI}), and anti-Pkb/Akt antibodies (αPkb). *B*, glucose uptake was measured in 3T3-L1 adipocytes treated with or without 20 μM JBP for 30 min before 10 min of stimulation with the indicated concentrations of insulin.

inhibiting JNK activation with a 12-amino acid peptide composed of the JNK-binding peptide identified in Jip1 (50). The JNK-binding peptide inhibits competitively the interaction of JNK with regulatory scaffolds or substrates. To facilitate translocation of the JNK-binding peptide (JBP) across the plasma membrane, the 12-amino acid transduction domain from human immunodeficiency virus TAT protein (HIV-TAT) was added to the N terminus (51). This chimeric peptide, TAT/JBP, was incubated with 3T3-L1 adipocytes for 30 min to disrupt JNK binding before insulin stimulation. TAT/JBP inhibited in a dose-dependent way insulin-stimulated JNK activity as assessed by c-Jun phosphorylation (Fig. 7*A*). TAT/JBP also blocked insulin-stimulated binding of JNK to Irs1 (Fig. 7*B*).

Inhibition of JNK Activity Reduced Ser^{307} Phosphorylation of Irs1 and Enhanced Insulin Signal Transduction and Glucose Uptake—To investigate the relation between JNK and Irs1, we treated 3T3-L1 adipocytes with the TAT/JBP and analyzed insulin-signaling events. TAT/JBP (20 μM) inhibited insulin-stimulated JNK activity and suppressed insulin-stimulated Ser^{307} phosphorylation of Irs1. Consistent with the inhibitory role of Ser^{307} phosphorylation, insulin-stimulated tyrosine phosphorylation of Irs1 increased (Fig. 8*A*). TAT/JBP also increased Akt phosphorylation during insulin stimulation (Fig. 8*A*). In 3T3-L1 adipocytes, insulin stimulates the uptake of 2-deoxyglucose by translocation of Glut4 to the plasma membrane (52). As expected, insulin stimulated uptake of 2-deoxyglucose into 3T3-L1 adipocytes. Incubation of 3T3-L1 adipocytes for 30 min with TAT/JBP but not the 12-residue HIV-TAT peptide increased insulin-stimulated glucose uptake (Fig. 8*B*). These results are consistent with the conclusion that JNK-mediated phosphorylation of Irs1 at Ser^{307} inhibits insulin action.

DISCUSSION

Our cell-based experiments reveal that recruitment of active JNK to Irs1 might be a common mechanism for feedback or heterologous inhibition of the insulin signal. JNK is a member of the MAP kinase family, which also includes the extracellular signal-regulated protein kinases (ERK1/2), p38 MAP kinases, and the ERK5 pathway (25). MAP kinases are activated by dual-specificity MAP kinase kinases that phosphorylate adjacent tyrosine and threonine residues (29). During insulin stimulation, ERK1 and ERK2 are strongly activated by the dual-specificity kinases MEK1 or MEK2, which are activated when Grb2/Sos bound to Irs1 or Shc activates the Ras → Raf cascade (53, 54); however, a molecular pathway linking the receptors for proinflammatory cytokines or insulin to JNK is difficult to establish.

JNK activation is best understood during cytokine stimulation, which involves the recruitment of an upstream multilineage kinase, a dual-specificity kinase (MKK4 or MKK7), and JNK into a regulatory complex including JNK-interacting protein Jip1 or Jip2 (49, 55). By contrast, previous studies suggest that insulin stimulates JNK through PI 3-kinase, Ras, or Shp2 (56). These distinct pathways might converge because the per-

meable JNK-binding peptide that disrupts TNFα-stimulated JNK activation also inhibits insulin-stimulated JNK; whether MLK and MKK4 or MKK7 are involved during insulin stimulation is unknown. Perhaps other kinase cascades are employed during insulin stimulation and are recruited with JNK by other scaffold proteins that contain a JNK-binding motif.

Irs1 is required for insulin stimulation of JNK in $32D^{IR}$ cells, because insulin barely stimulates JNK in these cells lacking Irs1. In $32D^{IR}$/Irs1 cells, the PI 3-kinase and the Ras $\rightarrow$ MEK1/2 $\rightarrow$ ERK1/2 cascades appear to be involved, because LY294002 or PD98059 significantly inhibit insulin-stimulated JNK activity. Although Irs1 contains a JNK-binding motif similar to that in Jip1 or Jip2, a direct interaction between JNK and Irs1 is not involved in the JNK activation during insulin stimulation. A mutant Irs1 protein lacking the JNK-binding motif mediates JNK activation normally in insulin-stimulated $32D^{IR}$ cells. By contrast, $F18^{Irs1}$, which lacks all the tyrosine phosphorylation sites but retains the JNK binding motif, fails to activate JNK during insulin stimulation and fails to bind inactive JNK. Thus, Irs1-mediated activation of the PI 3-kinase and Ras $\rightarrow$ MEK1/2 cascades is required for JNK activation, and Irs1 only binds activated JNK.

Our results with $32D^{IR}$ cells, MEFs, and 3T3-L1 adipocytes reveal that JNK is the principle kinase that mediates Ser^{307} phosphorylation during insulin stimulation. However, on inhibition of JNK through various strategies, some insulin-stimulated Ser^{307} phosphorylation still occurs. Many kinases are known to phosphorylate Irs1. Recent evidence indicates that IKKβ can directly phosphorylate Irs1 Ser^{307} (57). In addition, the possibility exists that mTOR phosphorylates Ser^{307}. We showed previously that insulin stimulates Ser^{307} phosphorylation in 3T3-L1 preadipocytes without activating JNK (24). Apparently, unknown differences in insulin signaling in preadipocytes disrupt the link between the insulin receptor and JNK, whereas other cell lines including 32D cells, MEF cells, and adipocytes show strong activation of JNK in response to insulin. Differential coupling between the insulin receptor and JNK might play an important role in feedback regulation of the insulin signaling cascade.

JNK has many effects on cellular function because it phosphorylates and activates various transcription factors, including ATF2 and ATFa, c-Jun and JunD, and Elk1 and Sap1 (28). Although insulin stimulation of JNK might play an important role in gene transcription as revealed in other systems, our results also suggest that JNK-mediated phosphorylation of Ser^{307} in rodent Irs1 mediates negative feedback of insulin signaling. Insulin stimulates the binding of activated JNK to Irs1 in 3T3-L1 and $32D^{IR}$/Irs1 cells, which attenuates insulin-stimulated tyrosine phosphorylation. Consistent with this hypothesis, disruption of the consensus JNK-binding motif in Irs1 significantly reduces the phosphorylation of Ser^{307} during insulin stimulation and increases insulin-stimulated tyrosine phosphorylation and Pkb/Akt activation. Moreover, inhibition of JNK activation by incubating 3T3-L1 adipocytes with a permeable TAT/JNK-binding peptide promotes insulin-stimulated glucose uptake. This result is consistent with reduced insulin-stimulated JNK activity and Ser^{307} phosphorylation and increased Irs1 tyrosine phosphorylation and Pkb/Akt phosphorylation. Experiments with mouse embryo fibroblasts lacking JNK1 and JNK2 also support these results. The possibility is not excluded that the binding of JNK to Irs1 itself might have some inhibitory effect in addition to Ser^{307} phosphorylation, such as competing with other proteins for binding to Irs1. Although our attention for the moment focuses on Ser^{307} phosphorylation, additional serine residues might also be involved. Moreover, we have not distinguished between JNK1 or JNK2 in our experiments, because both homologs bind to Irs1 (data not shown).

Insulin resistance and compensatory hyperinsulinemia dysregulate many physiological processes that contribute to life-threatening metabolic, vascular, and cardiac diseases (58, 59). Although new drugs are emerging to improve insulin sensitivity, the molecular mechanisms that cause insulin resistance are difficult to establish. The idea that inflammation causes insulin resistance has been known for a long time (60) and is consistent with the idea that anti-inflammatory drugs like high-dose aspirin promote insulin sensitivity (22, 61). The physiological response to infection, physical or thermal trauma, or obesity invariably involves the production of proinflammatory cytokines like TNFα that activate various serine kinases (28). Considerable evidence suggests that serine phosphorylation of the insulin receptor or the Irs proteins might inhibit insulin signaling and promote insulin resistance (62). During obesity, adipocytes produce TNFα, which promotes insulin resistance and stimulates serine phosphorylation of Irs1, whereas disruption of *TNFR1* partially restores insulin signaling and glucose tolerance in obese mice (18, 63–66). The signaling cascades regulated by TNFα are complex and involve many branch points, including the activation of JNK, p38, and the IKKβ (28). IKKβ might also be important because high doses of salicylates that inhibit IKKβ improve glucose tolerance in obese mice (22, 61). Salicylates increase insulin-stimulated phosphorylation of IRS proteins in the liver, revealing a potential mechanism for their effect on insulin action; the effect might occur indirectly through other downstream kinases, through NFκB-regulated gene expression. or through direct phosphorylation of Irs1 (57). By contrast, a direct role for JNK to regulate insulin signaling is compelling, because both Irs1 and Irs2 contain a JNK-binding motif. Other kinases might also bind to this motif and with JNK explain, at least in part, insulin resistance that occurs during trauma and obesity.

In summary, JNK mediates feedback inhibition of insulin signaling by phosphorylation of rat/mouse Irs1 at Ser^{307} (Ser^{312} in human Irs1). In addition to our experiments with $32D^{IR}$/Irs1 cells, MEF cells and 3T3-L1 adipocytes, insulin is reported to stimulate JNK activity in Rat-1 fibroblast, Chinese hamster ovary cells overexpressing human insulin receptors, L6 myotubes, and rat adipocytes (41–43). These results suggest that activated JNK might be an important negative feedback regulator for insulin signaling, and thus inhibiting JNK or interfering with JNK-Irs1 interaction might be a good therapeutic target to reduce insulin resistance.

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