Sequential phosphorylation of insulin receptor substrate-2 by glycogen synthase kinase-3 and c-Jun NH$_2$-terminal kinase plays a role in hepatic insulin signaling

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Sharfi H, Eldar-Finkelman H. Sequential phosphorylation of insulin receptor substrate-2 by glycogen synthase kinase-3 and c-Jun NH$_2$-terminal kinase plays a role in hepatic insulin signaling. Am J Physiol Endocrinol Metab 294: E307–E315, 2008. First published November 20, 2007; doi:10.1152/ajpendo.00534.2007.—Serine phosphorylation of insulin receptor substrate (IRS) proteins is a potential inhibitory mechanism in insulin signaling. Here we show that IRS-2 is phosphorylated by glycogen synthase kinase (GSK)-3. Phosphorylation by GSK-3 requires prior phosphorylation of its substrates, prompting us to identify the “priming kinase.” It was found that the stress activator anisomycin enhanced the ability of GSK-3 to phosphorylate IRS-2. Use of a selective c-Jun NH$_2$-terminal kinase (JNK) inhibitor and cells overexpressing JNK implicated JNK as the priming kinase. This allowed us to narrow down the number of potential GSK-3 phosphorylation sites within IRS-2 to four regions that follow the motif SXXXSP. IRS-2 deletion mutants enabled us to localize the GSK-3 and JNK phosphorylation sites to serines 484 and 488, respectively. Mutation at serine 488 reduced JNK phosphorylation of IRS-2, and mutation of each site separately abolished GSK-3 phosphorylation of IRS-2. Treatment of H4IIE liver cells with anisomycin inhibited insulin-induced tyrosine phosphorylation of IRS-2; inhibition was reversed by pretreatment with the JNK and GSK-3 inhibitors. Moreover, overexpression of JNK and GSK-3 in H4IIE cells reduced insulin-induced tyrosine phosphorylation of IRS-2 and its association with the p85 regulatory subunit of phosphatidylinositol 3-kinase. Finally, both GSK-3 and IRS-2 are insulin-resistant cell lines; insulin resistance.

THE INSULIN RECEPTOR SUBSTRATE (IRS) proteins are cytoplasmic adaptor proteins that mediate most, if not all, insulin signaling pathways. Of the six different IRS proteins identified, IRS-1 and IRS-2 participate in insulin-mediating mitogenetic growth and nutrient homeostasis (66). IRS-1/2 proteins become tyrosyl-phosphorylated by the insulin receptor (IR) tyrosine kinase and simultaneously initiate multiple signaling cascades via the recruitment of SH2-containing proteins, including the p85 regulatory subunit of phosphatidylinositol 3-kinase (PI3-kinase), Grb-2, SHP2, Nck, and Crk (37, 65). These signaling molecules activate downstream effectors that mediate the metabolic and growth stimulatory effects of insulin (37, 53, 65). Although the overall protein structures and amino acid sequences of IRS-1 and IRS-2 are similar (57), the proteins are not functionally interchangeable. This has been demonstrated by in vivo studies with knockout animal models that revealed differences in the signaling capacities of IRS-1 and IRS-2 (2, 28, 67, 70). IRS-1 and IRS-2 have different roles in insulin-mediated PI3-kinase/PKB activation, carbohydrate and lipid metabolism, cell growth, and cell differentiation (3, 6, 23, 46, 51, 58, 60). The two also have tissue-specific roles. For example, in muscle IRS-1 mediates glucose utilization and myoblast differentiation, whereas IRS-2 contributes to lipid metabolism and ERK signaling (5, 21, 23, 28, 46). In liver, IRS-2 prominently impacts gluconeogenesis and triglyceride production (28, 43, 46, 55, 58, 61). In adipose tissue, IRS-1 and IRS-2 exhibit different cellular compartmentalization and differentially regulate PI3-kinase, its downstream targets (PKB and PKC), and glucose transport (24, 49, 52, 54). Finally, recent studies implicate IRS-2 in a central role in regulating β-cell survival and β-cell mass (30, 59, 67). The fact that IRS-2 genetic deficiency in mice resulted in many of the features of type 2 diabetes (30, 67) suggested that dysregulation of IRS-2 represents an important mechanism responsible for human insulin resistance and type 2 diabetes (5, 36, 41). However, our understanding of the mechanisms mediating IRS-2 function is relatively poor.

Increasing evidence now suggests that serine/threonine phosphorylation of IRS-1 is a key player in negative-feedback regulation of insulin signaling. Studies detected enhanced in vivo serine phosphorylation of IRS-1 in diabetic tissues (4, 47, 50, 64). Multiple protein kinases were implicated in this process (1, 11, 20, 29, 33, 35, 48, 64); thus it is quite possible that serine phosphorylation of IRS-2 plays a negative role in insulin signaling, but this has not yet been conclusively demonstrated. In previous studies (32) we identified the serine/threonine protein kinase glycogen synthase kinase (GSK)-3 as an IRS-1 kinase that phosphorylates IRS-1 at serine 332. We proposed that GSK-3-mediated phosphorylation of IRS-1 contributes to insulin resistance, which was consistent with the view that GSK-3 is an important drug discovery target in insulin resistance and type 2 diabetes (14, 68). In the present research, we investigated the role of GSK-3 in the phosphorylation of IRS-2. The GSK-3 phosphorylation site in IRS-1, serine 332, is absent in IRS-2. Therefore, it was not possible to predict the GSK-3 IRS-2 phosphorylation site(s) by similarity. In other GSK-3 substrates, a consensus sequence follows the...
GSK-3 inhibits hepatic insulin signaling. Phosphorylates IRS-2 at serine 488 to allow GSK-3 phosphorylation of IRS-2 at serine 484. We further provide evidence that this sequential phosphorylation of IRS-2 by JNK/GSK-3 inhibits hepatic insulin signaling.

MATERIALS AND METHODS

Materials. Antibodies against IRS-2 and the p85 subunit of PI3-kinase were obtained from Upstate. Phospho-JNK (pThr183/pTyr185) was from Cell Signaling Technology. Anti-phosphotyrosine antibody PY99 and the anti-β-actin antibody were from Santa Cruz Biotechnology. Anti-Flag and -MAPKAP kinase 2 antibodies were available from the authors on request. The JNK-1 construct was a gift from Lilly Vardimon (Tel Aviv University; Ref. 45). The sequences of the mutagenic oligonucleotides are included.

Plasmids and transient transfection. The mouse IRS-2 gene in plasmid pMSCVpuro-IRS-2 was kindly provided by Dr. Renato Baserga (Thomas Jefferson University, Philadelphia, PA). IRS-2 fragments coding for amino acids 1–463, 1–498, 1–907, and 1–1018 were generated by PCR with respective primers and a GC-rich PCR system (Roche). PCR products were digested with HindIII and XhoI and then subcloned into the pCMV-Taq 2B expression vector. Mutations at serines 484 and 488 were introduced into the F2 construct with the QuickChange site-directed mutagenesis kit (Stratagene). All mutations and fragments were sequenced to confirm the presence of mutations. The sequences of the mutagenic oligonucleotides are available from the authors on request. The JNK-1 construct was a gift from Lilly Vardimon (Tel Aviv University; Ref. 45). The plasmid coding for GSK-3β was described previously (15).

Cells and transfections. HEK-293 cells or HepG2 hepatoma cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) containing 25 mM glucose, 10% fetal calf serum (FCS), 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Rat H4IE hepatoma cells were grown in α-MEM supplemented with 5% FCS and 5% horse serum. HEK-293 cells were transfected transiently with IRS-2 constructs (5 μg each) by the calcium phosphate method as previously described (15). Expression of wild-type (WT) IRS-2 or Flag-IRS-2 proteins was verified by Western blot analysis using specific antibodies against IRS-2 or Flag. H4IE cells were transfected transiently with JNK or GSK-3β constructs with an Easy-jet electroporator (EquiBio). Cells were treated either with anisomycin (20 μM) for 40 min or with the indicated inhibitor (10 μM) for 4 h before the addition of anisomycin.

Cell extraction and GSK-3 assay. Cells were collected and lysed in ice-cold buffer G (mM: 20 Tris pH 7.5, 10 β-glycerophosphate, 1 EGTA, 1 EDTA, 50 NaF, 5 NaPPi, 0.5 orthovanadate, and 1 benzamidine, with 10% glycerol, 5 μg/ml leupeptin, 25 μg/ml aprotinin, 5 μg/ml pepstatin, 500 mM dithiothreitol, and 1% Triton X-100). Cell extracts were centrifuged for 20 min at 15,000 g. Supernatants were collected, and equal amounts of cell lysates (200–300 μg protein as determined by Bradford assays) were subjected to immunoprecipitation with specific antibodies against IRS-2 or Flag as indicated. In some experiments, cells were treated with the inhibitors SP-600125 and SB-203580 (10 μM each, 4 h) before stimulation with anisomycin (20 μM, 40 min). GSK-3 kinase assays were performed in the immunoprecipitate complex. The reaction mixture contained 50 mM Tris (pH 7.4), 10 mM MgAc, [γ-32P]ATP (100 μM, 0.5 μCi/assay), 0.01% β-mercaptoethanol, and 10 mM microcystine and was incubated with purified recombinant GSK-3 (0.5 μg/reaction) for 20 min at 30°C. The reactions were stopped by the addition of boiled SDS sample buffer and subjected to gel electrophoresis. Gels were dried and autoradiographed. H4IE cells were pretreated with anisomycin (20 μM, 40 min) before the addition of insulin (10 nM, 90 s), or cells were treated with SP-600125 (10 μM, 4 h) and with the GSK-3-selective inhibitor L803-mts (44) (40 μM, 4 h) before the addition of anisomycin and incubation for an additional 40 min before stimulation with insulin (10 nM, 90 s). IRS-2 was immunoprecipitated from cell extracts and subjected to immunoblot analysis with anti-phosphotyrosine antibody PY99 or antibody against the p85 regulatory subunit of PI3-kinase.

Phosphorylation of IRS-2 by JNK. Flag-tagged WT F2 and its respective mutant F2S488A were immunoprecipitated from cell extracts with anti-Flag antibody. Purified JNK was added to the immunoprecipitates in a reaction mixture that contained 50 mM Tris (pH 7.3), 10 mM MgAc, and [γ-32P]ATP (100 μM, 0.5 μCi/assay) and was incubated for 20 min at 30°C. The reactions were stopped by the addition of boiled SDS sample buffer, and proteins were subjected to gel electrophoresis. Gels were dried and autoradiographed.

Sequential phosphorylation of IRS-2 by JNK and GSK-3. Flag-tagged F2 fragment was immunoprecipitated from cell extract with anti-Flag antibody as described above. Purified JNK (0.6 μg/reaction) was added to the immunoprecipitates in a reaction mixture that contained 50 mM Tris (pH 7.3), 10 mM MgAc, and 200 μM ATP and was incubated for 20 min at 30°C. The immunoprecipitates were washed twice to remove JNK, and purified GSK-3 (0.5 μg/reaction) was added to the immunoprecipitate together with the same reaction mixtures, except that [γ-32P]ATP (100 μM, 0.5 μCi/assay) was included, and the reaction was incubated for an additional 20 min. The reactions were stopped by the addition of boiled SDS sample buffer, and proteins were subjected to gel electrophoresis. Gels were dried and autoradiographed.

Animals and tissues. Ten-week-old Lep(ob/ob) male mice (referred to here as ob/ob) were purchased from Harlan. Lean (C57BL/6) and ob/ob mice were housed in individual cages in a temperature-controlled facility with a 12:12-h light-dark cycle. Animals had free access to water and were fed ad libitum. Animals were used at an age of 12 wk. Animal care and experimental protocols were approved by the Institutional Animal Care and Use Committee of Tel Aviv University. Animals were fasted for 4 h, and liver (lateral lobe) tissues from lean (C57BL/6) and ob/ob mice were homogenized with ice-cold buffer H (mM: 50 Tris pH 7.5, 150 NaCl, 1 EDTA, 1 EGTA, 10 β-glycerophosphate, and 50 NaF, with 5% glycerol, 1% Triton X-100, and 25 μg/ml each of 50 mM leupeptin, aprotinin, and pepstatin A). Homogenates were centrifuged at 10,000 g, and supernatants were collected. Equal amounts of protein lysate were subjected to SDS-PAGE followed by immunoblot analysis using antibodies as indicated in Figs. 1–5.

Densitometry and statistical analysis. Gel band quantitation was performed by densitometry analysis and carried out on exposures within the linear range. Graphics and statistical analyses were performed with Origin Professional 6.0 software. The significance of differences between experimental conditions was determined with the two-tailed Student’s t-test. Data were deemed significant when P < 0.05. Results are expressed as group means with SE. Band intensities of phosphorylated F1–F4 were determined (Fig. 3C). The “contaminated” band, which presented the autophosphorylated GSK-3, was subtracted from F1 and F2.

RESULTS

IRS-2 is phosphorylated by GSK-3. The ability of GSK-3 to phosphorylate IRS-2 was examined initially in HEK-293 cells that were transfected transiently with the mouse IRS-2 construct. IRS-2 protein was immunoprecipitated from the cell extracts and subjected to in vitro phosphorylation assays with GSK-3 (recombinant rabbit GSK-3β; Ref. 15). GSK-3 readily

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phosphorylated IRS-2 under these conditions (Fig. 1). IRS-2 was isolated from rat H4IIE liver cells or human hepatoma HepG2 cells as described above and subjected to in vitro phosphorylation assays with GSK-3. Both rat and human IRS-2 were phosphorylated by GSK-3 (Fig. 1). We concluded that the GSK-3 IRS-2 phosphorylation site(s) is likely conserved.

IRS-2 is primed by anisomycin and stress-activated kinase JNK. Mapping the GSK-3 phosphorylation site(s) on IRS-2 is a difficult task since IRS-2 is a very large protein (1,289 amino acids) and has many potential GSK-3 recognition sites: SXXXS(p). GSK-3 requires prior phosphorylation of its substrates at the P+4 position [S(p)] by a different kinase, also termed the priming kinase (18, 69). This requirement was demonstrated in GSK-3 phosphorylation of IRS-1 (32). We reasoned that identification of the nature of the priming kinase would narrow down the number of potential GSK-3 sites within IRS-2. Treatment of cells with various activators indicated that the stress activator anisomycin had the ability to prime IRS-2 for GSK-3 phosphorylation. In these experiments, HEK-293 cells, which overexpress IRS-2, or H4IIE hepatoma liver cells were treated with anisomycin for 40 min. IRS-2 was immunoprecipitated from cell extracts, and the immunoprecipitates were subjected to in vitro kinase assays with GSK-3. Pretreatment with anisomycin greatly enhanced the ability of GSK to phosphorylate IRS-2 in both cell lines (Fig. 2A), implying that an anisomycin-dependent kinase(s) primes IRS-2 for GSK-3. We decided to focus on two potential candidates that are activated by anisomycin, JNK, and p38 (7).

Experiments performed with JNK and p38 inhibitors SP-600125 and SB-203580, respectively, showed that pretreatment of H4IIE cells with SP-600125 before the addition of anisomycin greatly enhanced the ability of GSK to phosphorylate IRS-2 in both cell lines (Fig. 2A), implying that an anisomycin-dependent kinase(s) primes IRS-2 for GSK-3. We decided to focus on two potential candidates that are activated by anisomycin, JNK, and p38 (7).
anisomycin abolished the anisomycin-induced GSK-3 phosphorylation of IRS-2 (Fig. 2B). Treatment with SB-203580 had no effect (Fig. 2B). It was also shown that anisomycin activated both JNK isoforms as judged by the anti-phospho-JNK antibody (Fig. 2B). To verify that p38 was inhibited by SB-203580, we examined its effect on the p38 downstream target MAPKAP kinase-2. Pretreatment with SB-203580 partially inhibited the anisomycin-induced band shift of MAPKAP kinase-2, indicating that p38 was inhibited (Fig. 2B) as previously described (8). In addition, cells that overexpressed p38 did not enhance the ability of GSK-3 to phosphorylate IRS-2 (data not shown). Altogether, the results suggested that JNK is the priming kinase. Although we did not exclude the possibility that other anisomycin-dependent targets may be involved, we focused our further studies on JNK.

The role of JNK as the priming kinase was further examined in a cellular system. HEK-293 cells were cotransfected with plasmids coding for IRS-2 and WT JNK-1, and the phosphorylation of IRS-2 by GSK-3 was tested as described above. Overexpression of JNK-1 resulted in enhanced GSK-3 IRS-2 phosphorylation in anisomycin-stimulated conditions (Fig. 2C). The IRS-2 band shift observed in the JNK-1-expressing cells also demonstrated that JNK-1 phosphorylates IRS-2 under these conditions (Fig. 2C). We also observed an elevation in the phosphorylation levels of JNK-1 and its downstream target, c-Jun, further confirming that JNK-1 is active. As further proof, GSK-3 and JNK-1 were coexpressed with IRS-2 in HEK-293 cells. Expression of GSK-3 resulted in a small band shift of IRS-2, whereas expression of JNK-1 led to a much stronger effect (Fig. 2D). Expression of both GSK-3 and JNK-1 also resulted in a prominent band shift of IRS-2. We did not detect significant differences between IRS-2 band shifts observed in cells expressing JNK-1 alone or both JNK-1 and GSK-3. We think that this is likely due to the “small” contribution of GSK-3 to the “total” IRS-2 band shift. Alternatively, JNK could prime for endogenous GSK-3 that added to the detected band shift. Altogether, these results suggested that IRS-2 is a cellular target of the priming kinase JNK-1 and subsequently GSK-3.

Localization of GSK-3 phosphorylation site on IRS-2. IRS-2 contains multiple SXXXS motifs. However, JNK is a proline-directed kinase and typically phosphorylates an “XSP” motif. This directed our search toward SXXXSP motifs located within IRS-2. Four potential regions were identified: serines 381, 484, 961, and 976 as the GSK-3 sites and serines 385, 488, 965, and 1000 as the priming sites (Fig. 3A). Accordingly, four IRS-2 fragments were generated, termed here F1, F2, F3, and F4, coding for IRS-2 residues 1–463, 1–498, 1–907, and 1–1018, respectively (Fig. 3A). Notably, F1 and F2 included one and two sites, respectively. F3 included a JNK-binding motif (residues 881–887) that was previously shown to be essential for JNK phosphorylation of IRS-1 (31, 71). This motif (residues 881–887) that was previously shown to be essential for JNK phosphorylation of IRS-1 (31, 71). This motif (residues 881–887) that was previously shown to be essential for JNK phosphorylation of IRS-1 (31, 71).

Fig. 3. Localization of GSK-3 phosphorylation site at IRS-2. A: schematic presentation of generated fragments of IRS-2. The pleckstrin homology (PH), phosphotyrosine binding (PTB), and kinase regulatory loop binding (KRLB) domains are shown. The 4 motifs of SXXXSP are indicated and termed sites 1–4. The location of the JNK binding motif to IRS-2 (determined by similarity) is also indicated. B: Flag-IRS-2 fragments (F1–F4) were expressed in HEK-293 cells and were detected by immunoblot analysis with anti-Flag antibody. The apparent molecular mass (kDa) of the expressed proteins is indicated. C: IRS-2 fragments shown in B were immunoprecipitated from cell extracts with anti-Flag antibody, and the immunoprecipitates were subjected to in vitro phosphorylation assays with GSK-3 using [γ-32P]ATP as described in MATERIALS AND METHODS. Molecular mass of the phosphorylated proteins is indicated. Autophosphorylation of recombinant GSK-3 appears as a 68-kDa band. D: HEK-293 cells expressing F1 or F2 were treated with or without anisomycin. Phosphorylation of IRS-2 fragments by GSK-3 was determined as described in C. Expression of fragments from whole cell lysates is shown at bottom. For B–D, a representative gel from 1 of 3 independent experiments is shown.
fragment was generated because JNK may require this motif for phosphorylation of F1 and F2. The F4 fragment included all four possible sites. The constructs were expressed as Flag-tagged proteins in HEK-293 cells (Fig. 3B). In the next step, the proteins were immunoprecipitated with anti-Flag antibody from cell extracts and were subjected to in vitro phosphorylation assays with GSK-3, as described above. The results indicated that F2, but not F1, was phosphorylated by GSK-3 (Fig. 3C). The phosphorylation of F3 and F4 by GSK-3 was comparable with that of F2 (as concluded by densitometry analysis; see MATERIALS AND METHODS). This suggested that F2 contains the GSK-3 phosphorylation site (site 2 in Fig. 3A), which is likely the sole GSK-3 phosphorylation site of IRS-2. To further confirm this conclusion and to eliminate a possible role for site 1, cells expressing F1 or F2 were treated or not treated with anisomycin. IRS-2 proteins were immunoprecipitated from cell extracts with anti-Flag antibody and subjected to in vitro phosphorylation assays with GSK-3. Clearly, F1 was not phosphorylated by GSK-3, even after stimulation with anisomycin (Fig. 3D). This suggested that the GSK-3 phosphorylation site resides within residues 463–498 and is likely serine 484, lying in the context SAGSGP.

GSK-3 and JNK phosphorylate IRS-2 at serines 484 and 488, respectively. The results presented so far led us to conclude that serine 484 is the GSK-3 phosphorylation site and serine 488 is the priming site phosphorylated by JNK. To verify these assumptions, serines 484 and 488 were mutated separately to alanine in the context of the F2 fragment, generating two respective mutants, F2S484A and F2S488A. F2 and these mutants were expressed transiently in HEK-293 cells.

The proteins were immunoprecipitated with anti-Flag antibody and subjected to GSK-3 phosphorylation assays as described above. As shown in Fig. 4A, GSK-3 failed to phosphorylate F2S484A and F2S488A mutants, even after stimulation with anisomycin. This clearly indicated that serines 484 and 488 are involved in GSK-3 phosphorylation. The ability of JNK to phosphorylate the F2S488A mutant was examined next. F2 and the F2S488A mutant were incubated with purified active JNK. JNK phosphorylated F2, but its ability to phosphorylate F2S488A was reduced relative to phosphorylation of F2 (reduction of 45% ± 4) (Fig. 4B). This indicated that serine 488 is indeed a JNK phosphorylation site but is likely not the only site. Indeed, recent work reports that threonine 348 is a JNK phosphorylation site (56). Finally, we demonstrated the sequential phosphorylation of IRS-2 by JNK/GSK-3. In this experiment, F2 was first phosphorylated by purified active JNK with “cold” ATP. The enzyme then was removed from the reaction mixture, and GSK-3 kinase assays were performed with “hot” ATP. Clearly, pretreatment with JNK enhanced the ability of GSK-3 to phosphorylate the protein (Fig. 4C). In summary, we identified GSK-3’s phosphorylation site as serine 484 and demonstrated sequential phosphorylation of IRS-2 by JNK and GSK-3 at serines 488 and 484, respectively.

JNK and GSK-3 inhibit insulin-mediated IRS-2 signaling in liver cells. To explore the role of serine phosphorylation of IRS-2 in the insulin signaling pathway, H4IIE liver cells were treated with anisomycin before stimulation with insulin and tyrosine phosphorylation of IRS-2 was examined. Treatment with anisomycin significantly reduced insulin-induced tyrosine phosphorylation of IRS-2 was examined. Treatment with anisomycin significantly reduced insulin-induced tyrosine phosphorylation of IRS-2 was examined. Treatment with anisomycin significantly reduced insulin-induced tyrosine phosphorylation of IRS-2 was examined.
phosphorylation of IRS-2 and its association with the p85 regulatory subunit of PI3-kinase (Fig. 5A). When cells were treated with JNK inhibitor, SP-600125, GSK-3 inhibitors, L803-mts (44), or with combination of both inhibitors, this inhibitory effect was partially restored (Fig. 5B). It is noteworthy that the anisomycin-IRS-2 band shift was reduced by the treatment with both inhibitors (Fig. 5B). Next, H4IIE cells were transfected transiently with JNK or JNK + GSK-3β plasmids, and their impact on tyrosine phosphorylation of IRS-2 was determined. Overexpression of JNK and GSK-3β in H4IIE cells resulted in a significant reduction in insulin-induced tyrosine phosphorylation of IRS-2 and its association with the p85 regulatory subunit of PI3 kinase (Fig. 5C). Therefore, JNK and GSK-3 inhibit IRS-2-transduced insulin signaling through phosphorylation of IRS-2. We predicted that either GSK-3 or JNK or both are abnormally regulated in the

**Fig. 5. JNK/GSK-3 impair insulin action in liver cells.** A: H4IIE liver cells were treated with anisomycin before stimulation with insulin (10 nM, 90 s) as described in MATERIALS AND METHODS. IRS-2 proteins were immunoprecipitated (IP) from cell extracts and subjected to immunoblot (IB) analysis with anti-phosphotyrosine (pY) antibody or a p85 regulatory subunit antibody. Tyrosine phosphorylated IRS-2 is indicated. Association of IRS-2 with the p85 regulatory subunit of PI3-kinase is shown at bottom. Densitometry analysis of p85 bands is shown as fold of insulin-treated (designated 1) (right). Results are means ± SE of 3 experiments; *P* < 0.05 for Aniso-insulin vs. insulin. B: H4IIE cells were treated with the JNK inhibitor SP-600125, GSK-3 inhibitor, L803-mts, or combination of both inhibitors before addition of anisomycin and stimulation with insulin as described in MATERIALS AND METHODS. IRS-2 tyrosine phosphorylation was determined as described in A. Association of IRS-2 with p85 regulatory subunit and expression levels of JNK-1, GSK-3β/α, and β-actin from whole cell lysates are shown at bottom. Densitometry analysis of tyrosine phosphorylated IRS-2 bands is shown as fold of insulin-treated (designated 1) (right). Treatments: insulin (*a*), anisomycin (*b*), L803-mts (*c*), SP600125 (*d*), SP600125+L803-mts (*e*). Results are means ± SE of 3 experiments; *P* < 0.05 for *c–e* vs. *b*. C: H4IIE cells were transiently transfected with JNK-1 or with JNK-1 and GSK-3β constructs. Cells were treated with insulin (10 nM, 90 s), and tyrosine phosphorylation of IRS-2 was detected as described in A. Expression levels of JNK-1, GSK-3β, and β-actin from whole cell lysates are shown at bottom. Densitometry analyses of tyrosine phosphorylated IRS-2 and p85 bands are shown as fold of insulin-treated (designated 1) (right). Treatments: insulin (*a*), JNK (*b*), JNK+GSK-3β (*c*). Results are means ± SE of 3 experiments; *P* < 0.05 for *b* and *c* vs. *a*. D: liver tissue extracts prepared from lean or ob/ob mice were subjected to gel electrophoresis followed by immunoblot analysis with antibody against IRS-2, GSK-3β, JNK, and anti-phospho-JNK as indicated. Representative results from 3 animals (of 6) are shown. For A–D, a representative gel from 1 of 3 independent experiments is shown.
diabetic liver. The abundance of JNK and GSK-3 was examined in tissue extracts of liver from lean or diabetic/obese (ob/ob) animals (72). As hypothesized, both JNK-1 and GSK-3β were remarkably elevated in the ob/ob livers compared with their control counterparts (Fig. 5D). Enhanced phosphorylation of JNK-1 further indicated that JNK is hyperactive in the ob/ob liver (Fig. 5D).

DISCUSSION

Our results support a previously unknown inhibitory mechanism of insulin signaling via serine phosphorylation of IRS-2. Our novel findings showed that 1) JNK phosphorlates IRS-2 at serine 488 and serves as the priming kinase for GSK-3, 2) GSK-3 phosphorlates IRS-2 at serine 484, 3) both serine sites are highly conserved among mouse, rat, and human (Fig. 4D), and 4) JNK/GSK-3-mediated phosphorylation of IRS-2 inhibits its insulin signaling in liver cells. We further showed that the expression levels of JNK and GSK-3 are remarkably elevated in the diabetic liver (Fig. 5D).

JNK and GSK-3 are important mediators in insulin resistance, and both are potential therapeutic targets for treatment of obesity and type 2 diabetes. JNK activity and/or expression levels are elevated in obese and diabetic animals and were shown to contribute to development and progression of insulin resistance (22, 26, 31). Genetic manipulations in JNK activity further verified its causative role in insulin resistance. Mice lacking JNK-1 exhibited decreased adiposity, significantly improved insulin sensitivity, and enhanced insulin receptor signaling capacity (22). Conversely, adenoviral JNK-1 overexpression in the liver of normal mice increased expression of gluconeogenic enzymes phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase and decreased "whole liver" expression in the liver of normal mice increased expression of gluconeogenic enzymes (25, 32, 34).

A common mechanism that links JNK and GSK-3 with insulin resistance is their ability to phosphorylate IRS-1 (at serines 307 and 332, respectively) and to generate a negative-feedback regulation of insulin receptor signaling (1, 32). Here we show that GSK-3 and JNK also phosphorylate IRS-2. However, unlike phosphorylation of IRS-1, which is independently phosphorylated by JNK and GSK-3, IRS-2 is subject to cooperative/sequential phosphorylation by JNK and GSK-3 that enhances the inhibitory impact. It is also plausible that the different serine phosphorylation patterns observed for IRS-1 and IRS-2 determine their diverse functions in insulin signaling in various tissues and are dependent on the nature of protein kinases that catalyze these phosphorylation events.

It is noteworthy that JNK is the priming kinase for GSK-3. Other priming kinases were reported previously, including casein kinase-2, PKA, and ERK (9, 19, 69). This newly revealed mechanism establishes a previously unsuspected link between GSK-3 and stress activation. How phosphorylation at serine inhibits IRS-2 function is not known at this point. Interestingly, these sites are in proximity to the IRS-2-phosphotyrosine binding (PTB) domain, which was shown to facilitate the IRS-1 interaction with the insulin receptor (13, 62). It is thus quite possible that phosphorylation of these sites disrupts interaction of IRS-2 and the insulin receptor. It is also possible that phosphorylation of IRS-2 may recruit signaling molecules that sterically inhibit the interactions of IRS-2 and the NPEY motif of the insulin receptor, preventing tyrosine phosphorylation of IRS-2 (35).

In summary, we present a novel mechanism of sequential phosphorylation of IRS-2 at serines 488 and 484 by JNK and GSK-3, respectively, and demonstrate the inhibitory role of this phosphorylation in insulin signaling.

GRANTS

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