Modulation of Insulin-stimulated Degradation of Human Insulin Receptor Substrate-1 by Serine 312 Phosphorylation*

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Ser/Thr phosphorylation of insulin receptor substrate-1 (IRS-1) is a negative regulator of insulin signaling. One potential mechanism for this is that Ser/Thr phosphorylation decreases the ability of IRS-1 to be tyrosine-phosphorylated by the insulin receptor. An additional mechanism for modulating insulin signaling is via the down-regulation of IRS-1 protein levels. Insulin-induced degradation of IRS-1 has been well documented, both in cells as well as in patients with diabetes. Ser/Thr phosphorylation of IRS-1 correlates with IRS-1 degradation, yet the details of how this occurs are still unknown.

In the present study we have examined the potential role of different signaling cascades in the insulin-induced degradation of IRS-1. First, we found that inhibitors of the phosphatidylinositol 3-kinase 3-kinase and mammalian target of rapamycin block the degradation. Second, knockout cells lacking one of the key effectors of this cascade, the phosphoinositide-dependent kinase-1, were found to be deficient in the insulin-stimulated degradation of IRS-1. Conversely, overexpression of this enzyme potentiated insulin-stimulated IRS-1 degradation. Third, concurrent with the decrease in IRS-1 degradation, the inhibitors of the phosphatidylinositol 3-kinase and mammalian target of rapamycin also blocked the insulin-stimulated increase in Ser312 phosphorylation. Most important, an IRS-1 mutant in which Ser312 was changed to alanine was found to be resistant to insulin-stimulated IRS-1 degradation. Finally, an inhibitor of c-Jun N-terminal kinase, SPP00125, at 10 µM did not block IRS-1 degradation and IRS-1 Ser312 phosphorylation yet completely blocked insulin-stimulated c-Jun phosphorylation. Further, insulin-stimulated c-Jun phosphorylation was not blocked by inhibitors of the phosphatidylinositol 3-kinase and mammalian target of rapamycin, indicating that c-Jun N-terminal kinase is unlikely to be the kinase phosphorylating IRS-1 Ser312 in response to insulin. In summary, our results indicate that the insulin-stimulated degradation of IRS-1 via the phosphatidylinositol 3-kinase pathway is in part dependent upon the Ser312 phosphorylation of IRS-1.

The first step in insulin action is ligand stimulation of the insulin receptor tyrosine kinase. A number of endogenous substrates, including insulin receptor substrates (IRS)1–4 (1–4) are phosphorylated on tyrosine residues. Tyrosine-phosphorylated IRS-1 and IRS-2 serve as the major docking proteins for Src homology 2 domain containing proteins (5, 6). Association of the p85 regulatory subunit of phosphatidylinositol (PI) 3-kinase with tyrosine-phosphorylated IRS-1/2 results in the membrane localization and activation of the p110 catalytic subunit of PI 3-kinase, leading to the generation of phosphatidylinositol 3,4,5-trisphosphate and phosphatidylinositol 3,4-bisphosphate. These lipid products induce the activation of a number of signaling kinases including the Ser/Thr kinase Akt (7, 8), which is phosphorylated and activated by the upstream PDK1 kinase (9). Activation of Akt and its downstream signals have been shown to play a critical role in mediating the metabolic actions of insulin such as GLUT4 translocation and glucose transport (10–13), glycogen synthesis kinase 3 serine phosphorylation and glycogen synthesis (14), phosphodiesterase serine phosphorylation and antilipolysis (15–17), and mTOR activation and protein synthesis (18–21). However, other effects of insulin, for example its mitogenic actions, are mediated by association of Grb2 with tyrosine-phosphorylated Shc and IRS-1/2, which results in the activation of the mitogen-activated protein kinase signaling pathway (5, 6).

In addition, other signaling cascades stimulated by insulin may also participate in these responses (22).

Some of the same signaling molecules that are involved in the metabolic and mitogenic actions of insulin have also been proposed to play a role in both the feedback inhibition of the insulin signal and cellular insulin resistance (23–25). It has been proposed that hyper-Ser/Thr phosphorylation of IRS proteins plays a key role in the uncoupling of the insulin signal. Hyperphosphorylation of IRS-1 on Ser/Thr residues has been shown in both cultured cells and in vivo to be associated with an insulin-resistant state (26, 27). Densensitization of the insulin signal via IRS-1 Ser/Thr phosphorylation can result from counterregulatory hormone activation, proinflammatory cytokine production/cellular stress, or inhibition of protein kinases 1 and 2A (26, 28–31). Several specific phosphorylation sites have been identified as targets of these counter regulatory hormones and their signaling cascades. Activation of the mitogen-activated protein kinase signaling pathway has been shown to result in an increase in the phosphorylation of Ser312 of IRS-1 (32, 33),2 whereas activation of the JNK has

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‡ The abbreviations used are: IRS, insulin receptor substrate; PI, phosphatidylinositol; PDK1, phosphoinositide-dependent kinase 1; wt, wild type; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; mTOR, mammalian target of rapamycin; MEK, mitogen-activated protein kinase/extracellular signal regulated kinase kinase; ES, embryonic stem.

† Unless otherwise specified, the sequence numbers shown correspond to the human sequence of IRS-1. Thus, Ser316 corresponds to rat Ser312 and Ser312 corresponds to rat Ser317.
Ser312 was replaced with Ala was decreased compared with the wt IRS-1. In contrast, a mutant IRS-1 in which Ser 616 was changed to Ala exhibited insulin-induced degradation compared with the wt IRS-1. Insulin-stimulated IRS-1 degradation and Ser312 phosphorylation was partially blocked with a JNK small molecule inhibitor. However, subsequent studies showed that the insulin-stimulated phosphorylation of Ser312 was unlikely to be due to JNK because other inhibitors (like LY294002) could also block this phosphorylation without inhibiting JNK. These results indicate that phosphorylation at Ser312 is necessary for insulin-stimulated degradation and suggests that phosphorylation at Ser312 has a dual mechanism of modulating insulin action: uncoupling the interaction of IRS-1 with the insulin receptor as well as the targeting of IRS-1 to the degradation pathway.

**EXPERIMENTAL PROCEDURES**

Materials—Polyclonal antibodies to IRS-1, Ser(P)Tyr312, IRS-1, IRS-2, and PI 3-kinase (p85) were from Upstate Biotechnology (Lake Placid, NY). Polyclonal anti-Ser(P)Tyr616 IRS-1 antibody was from BIOSOURCE (Camarillo, CA). Monoclonal anti-Ser(P)Tyr616 c-Jun antibody was from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal anti-Thr(P)Ser202/Thr204 ERK1/2 was from Cell Signaling (Beverly, MA). Monoclonal anti-Akt1 antibody was from Transduction Laboratories (San Diego, CA). Monoclonal anti-IRS-1 antibody (1D6) was used as shown (28). Polyclonal anti-ERK1/2 antibody (DC3) was a gift from James E. Ferrer, Jr. (Stanford University, Stanford, CA). Cloning enzymes and competent DH5α cells were from Invitrogen. Plasmid purification kits were from Qiagen. Turbo DNA polymerase, Turbo Exonuclease I, T4 DNA ligase, Recombinant T4 DNA polymerase, and restriction enzymes and ligated into pWZL zeocin by digestion with EcoRI and XbaI-digested pcDNA3.1 (+) zeocin. Ser616 or Ser312 in human IRS-1 was mutated to Ala or Ser616 or Ser312, respectively, with the QuikChange XL mutagenesis kit from Stratagene (La Jolla, CA). Enhanced chemiluminescence detection reagents were from Pierce. Porcine insulin, goat anti-mouse, and anti-rabbit peroxidase-conjugated antibodies, monoclonal anti-FLAG, anti-FLAG-agarose, and other chemicals were from Sigma. LY294002, SP600125, rapamycin, U0126, MG132, and synthetic lactacystin were from Calbiochem (San Diego, CA). Tri-glycine gels, NuPAGE gels, and electrophoresis reagents were from Novex/Invitrogen.

Plasmid Construction and Mutagenesis—C terminus epitope-tagged human IRS-1 was subcloned from pFastBac (53) using EcoRI and XbaI, and EcoRI restriction enzymes and ligated into EcoRI- and XbaI-digested pcDNA3.1 (+) zeocin. Ser312 or Ser616 in human IRS-1 was mutated to Ala (S616A or S312A, respectively) with the QuikChange XL mutagenesis kit using the sense primer 5′-TACATGGCATATGCCAGGGGTGG-3′ and antisense primer 5′-CCACCCCTGGGCAATGGCAGTGCTG-3′.

Generation of Stable Cell Lines Expressing the Wild Type and Mutant IRS-1 and PKD1—H4IIErat hepatoma cells were infected with pWZL expressing IRS-1 or PKD1 constructs as described (55), with slight modifications. Briefly, 85% confluent Phoenix packaging cells were transfected using FuGENE 6 (Roche Molecular Biochemicals) or LipofectAMINE (Invitrogen) according to the manufacturer’s instructions. 24 h post-transfection, fresh medium was added and allowed to incubate at 37 °C for 24 h to generate viral supernatant. 50–60% confluent H4IE cells were incubated with viral supernatant for 24 h at 37 °C. Stable cell pools expressing specific IRS-1 were generated by selection for 48 h with 5 μg/ml blastocidin HC1 and then maintained in 1 μg/ml blastocidin HC1. Stable cell pools expressing PKD1 were selected with 500 μg/ml hygromycin.

Cell Culture and Treatments—H4IE cells were maintained in Dulbecco’s modified Eagle’s medium containing 5% fetal bovine serum, 5% newborn calf serum at 37 °C and 5% CO2 and then serum deprived for 10–18 h in Dulbecco’s modified Eagle’s medium containing 0.2% bovine serum albumin. The cells were pretreated with various inhibitors or Me6SO for 30 min prior to insulin stimulation. Insulin or 0.01 nM HCl was added at the time of serum deprivation as described in the figure legends. Mouse embryonic stem (ES) cells lacking PKD1 and control ES cells were cultured as previously described (56, 57). ES cells were serum deprived for 3 h in Dulbecco’s modified Eagle’s medium containing 0.5% bovine serum albumin and 20 μM Hesperin prior to insulin stimulation as described in the figure legends.

Immunoprecipitation and Immunoblotting—H4IE cells were lysed
by shaking on ice for 20 min with 50 mM Hepes, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 1 mM Na3VO4, 100 mM okadaic acid, and 1× protease inhibitor set I mixture (Calbiochem). Cellular debris was removed by centrifugation at 15,000 rpm for 15 min at 4 °C. The protein content was determined by the BCA assay. Approximately 20–30 and 200–300 μg of protein were used for analyses of total cell lysates and immunoprecipitations, respectively. Immunoprecipitations were performed with 30 μl of anti-FLAG agarose at 4 °C for 3 h. Immunoprecipitated IRS-1 proteins were washed with 20 mM Tris, pH 7.4, 200 mM NaCl, 0.1% Triton X-100; then with 50 mM Tris, pH 7.4, 500 mM NaCl; then with 50 mM Tris, pH 7.4, 150 mM NaCl; and then with 20 mM Tris, pH 7.4, 200 mM NaCl. Agarose beads were resuspended in 1× sample buffer (58 mM Tris, pH 6.8, 1% SDS, 40% glycerol, and 0.1 mM dithiothreitol), H4PDK1 and ES cells were lysed on ice with buffer B (50 mM Tris, pH 7.5, 150 mM NaCl, 1% Triton X-100, 10% glycerol, 1 mM EDTA, 1 mM dithiothreitol, 1 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride, 1 mM leupeptin, 10 μg/ml aprotinin, 30 mM NaPPi, 1 mM Na3VO4, and 100 mM okadaic acid). Cellular debris was removed by centrifugation at 15,000 rpm for 15 min at 4 °C. The immunoprecipitations were performed with 2 μl of anti-IRS-1 antibody (ID6) at 4 °C for 3 h, followed by collection on protein G-Sepharose. Immunoprecipitated IRS-1 proteins were washed three times with buffer B. Sepharose beads were resuspended in 1× sample buffer. The samples were boiled for 5 min and subjected to SDS-PAGE using 6%, 7.5%, or 10% Tris-glycine gels, then transferred to nitrocellulose, and blotted with anti-IRS-1 or anti-p85 antibodies. A, insulin-induced IRS-1 degradation in the H4 cells. H4 wt IRS-1 cells were treated with various doses of insulin for 18 h in serum-free medium. Representative blots from at least three independent experiments are shown. B, proteasome inhibitors block the insulin-induced IRS-1 degradation in H4 cells. H4IIE cells and H4 wt IRS-1 cells were pretreated with 15 μM MG132 or 5 μM lactacystin and then stimulated with 20 nM insulin for 8 h at 37 °C. Shown are representative blots from two independent experiments. IB, immunoblot.

**Fig. 1.** Dose-dependent insulin-stimulated degradation of expressed IRS-1 in H4IIE cells and the effect of proteasome inhibitors. H4IIE cells were infected with a retrovirus encoding a FLAG-tagged wild-type human IRS-1 (H4 wt IRS-1) and drug-selected to isolate a pool population of cells stably expressing the constitut. The cells were lysed, and the total cell lysates or anti-FLAG immunoprecipitates were separated on 6% SDS-PAGE gels, transferred to nitrocellulose, and blotted with anti-IRS-1 or anti-p85 antibodies. A, insulin-induced IRS-1 degradation in the H4 cells. H4 wt IRS-1 cells were treated with various doses of insulin for 18 h in serum-free medium. Representative blots from at least three independent experiments are shown. B, proteasome inhibitors block the insulin-induced IRS-1 degradation in H4 cells. H4IIE cells and H4 wt IRS-1 cells were pretreated with 15 μM MG132 or 5 μM lactacystin and then stimulated with 20 nM insulin for 8 h at 37 °C. Shown are representative blots from two independent experiments. IB, immunoblot.

**RESULTS**

**Insulin Stimulates IRS-1 Degradation.**—Insulin-stimulated degradation of IRS-1 has been reported in a number of cellular models of insulin resistance (42, 46, 47, 58). To determine whether IRS-1 protein levels are regulated by insulin in the highly responsive rat hepatoma H4IIE cells (59), cells stably expressing FLAG-tagged wild-type human IRS-1 (H4 wt IRS-1) were stimulated with various concentrations of insulin for 18 h. The cell lysates were analyzed by SDS-PAGE followed by transfer to nitrocellulose and immunoblotting with anti-IRS-1 antibodies. A dose-dependent decrease in IRS-1 protein levels was detected with a 60 ± 4% decrease at 10 nM insulin (Fig. 1A). A control blot of the p85 subunit of PI 3-kinase in total cell lysates showed no decrease under the same conditions (Fig. 1A). To confirm the role of intracellular proteolysis in this insulin-stimulated IRS-1 degradation, H4 wt IRS-1 cells were pretreated with or without MG132 and lactacystin, specific inhibitors of the 26 S proteasome. Consistent with other cellular models of insulin-stimulated IRS-1 degradation (42, 46, 47, 58), MG132 and lactacystin completely blocked insulin-stimulated degradation (Fig. 1B, lanes 5 and 7 versus lane 3). The ability of MG132 alone to stimulate a shift in the molecular weight of the IRS-1 (Fig. 1B) indicates that it is likely activating the stress-activated kinases in these cells. The lack of an IRS-1 band in the anti-FLAG immunoprecipitates from the parental H4IIE cells (Fig. 1B, lane 1) verifies that the band being detected is the expressed human IRS-1.

**Modulation of Insulin-stimulated IRS-1 Degradation by the PI3 Kinase/Akt/mTOR Pathway.**—To investigate the signaling pathways mediating insulin-stimulated IRS-1 degradation in the H4IIE cells, H4 wt IRS-1 cells were pretreated with various...
inhibitors for 30 min and then stimulated with 10 nM insulin for 18 h. The cell lysates were analyzed by SDS-PAGE followed by transfer to nitrocellulose and immunoblotting. In the absence of any inhibitor, a 60% reduction in IRS-1 protein levels was detected with insulin stimulation. In the presence of the PI 3-kinase inhibitor LY294002, no IRS-1 degradation occurred (Fig. 2, A and C). Treatment of the H4 cells with this drug actually increased the IRS-1 levels, suggesting that even the basal turnover of IRS-1 was being inhibited. To determine whether downstream members of this pathway are involved, rapamycin, an inhibitor of mammalian target of rapamycin (mTOR), was tested for its effect. Rapamycin also completely blocked insulin-stimulated IRS-1 degradation (116 ± 2% of nontreated, n = 4). Controls verified that at the concentrations used, rapamycin and LY294002 completely inhibited the insulin-stimulated mobility shift in p70 S6 kinase (Fig. 2A). In contrast to these results, the MEK1/2 inhibitor UO126 did not inhibit (p = 0.48, n = 3) insulin-stimulated IRS-1 degradation (Fig. 2, A and C). As a control, the effect of pretreatment with UO126 on insulin-stimulated ERK1/2 activation was assessed. Insulin stimulated a ~18-fold increase in ERK1/2 (Thr^{202}/Tyr^{204}) phosphorylation that was completely blocked by pretreatment with UO126 (Fig. 2B), whereas no inhibition of the insulin-stimulated shift in p70 S6 kinase was observed.

To assess what effects these inhibitors had on the specific phosphorylation sites in IRS-1, the IRS-1 from these cells was probed with anti-Ser(P)312 and anti-Ser(P)616. Intriguingly, the insulin-stimulated increase in Ser^{312} phosphorylation was reduced 90% and 78% by LY294002 and rapamycin pretreatment, respectively (Fig. 2, A and D). On the other hand, the MEK inhibitor had a greater ability to inhibit Ser^{616} phosphorylation (66 ± 17%). This is consistent with the prior data that identified this site as a mitogen-activated protein kinase-regulated Ser phosphorylation site (32, 33). In contrast to the results on Ser^{312}, the insulin-stimulated increase in Ser^{616} phosphorylation (~5-fold) was not significantly reduced by LY294002 and rapamycin pretreatment (74 ± 35 and 75 ± 16% of nontreated, respectively) (Fig. 2D).
Because chemical inhibitors may act on undefined targets in cells in addition to their intended targets, we sought to further test the role of the PI 3-kinase cascade in the insulin-stimulated degradation of IRS-1. As a first step, we overexpressed PDK1 in the H4IIE cells. As expected, these cells showed an increase in response to the insulin-stimulated phosphorylation of Akt on Thr\(^{308}\), the PDK1 phosphorylation site (60, 61). More importantly for the purposes of these studies, the H4IIE cells overexpressing PDK1 showed an increased sensitivity in the insulin-stimulated degradation of IRS-1 (Fig. 3A). To further test the role of this pathway in the insulin-stimulated degradation of IRS-1, we then examined the ability of insulin (and insulin-like growth factor-I) to stimulate IRS-1 degradation in ES cells lacking PDK1 (57). In comparison with the parental
PDK1 cells, insulin and insulin-like growth factor I stimulated a much lower amount of IRS-1 degradation in these cells (Fig. 3B).

The Role of Ser\textsuperscript{616} in Insulin-stimulated IRS-1 Degradation—The above results strongly support the role of the PI 3-kinase/Akt/mTOR pathway in the insulin-stimulated degradation of IRS-1. To determine the role of specific Ser phosphorylation sites in the insulin-stimulated degradation of IRS-1, we then assessed the ability of insulin to stimulate the degradation of various mutant IRS-1 molecules. As noted above, insulin greatly stimulated the phosphorylation of Ser\textsuperscript{616}. The human IRS-1 Ser\textsuperscript{616} was mutated to Ala (S616A), and stable H4IIE cells expressing S616A IRS-1 (H4 S616A IRS-1) were generated as described under “Experimental Procedures.” H4IIE cells stably expressing either the wt IRS-1 or S616A IRS-1 were stimulated with 10 nM insulin for 18 h. Anti-FLAG immunoprecipitates and cell lysates were analyzed by SDS-PAGE followed by transfer to nitrocellulose and immunoblotting. Both wild type IRS-1 and the S616A mutant were comparably degraded after stimulation with insulin (62 ± 4 and 49 ± 9% of nontreated, respectively) (Fig. 4). The specificity of the anti-Ser(P)\textsuperscript{616} antibody was confirmed by the finding that the IRS-1 S616A mutant protein only weakly reacted with this antibody compared with wild type IRS-1 (Fig. 4A). These results suggest that phosphorylation at Ser\textsuperscript{616} does not play a major role in insulin-stimulated IRS-1 degradation.

The Role of Ser\textsuperscript{312} in Insulin-stimulated IRS-1 Degradation—In addition to Ser\textsuperscript{616}, insulin also stimulates the phosphorylation of IRS Ser\textsuperscript{312}. However, in contrast to the insulin-stimulated phosphorylation of IRS-1 on Ser\textsuperscript{616}, IRS-1 Ser\textsuperscript{312} phosphorylation was inhibited by the same agents (LY294002 and rapamycin) that inhibited the insulin-stimulated IRS-1 degradation. To directly test the potential role of Ser\textsuperscript{312} phosphorylation in insulin-stimulated IRS-1 degradation, this Ser was mutated to Ala, and H4IIE cells stably overexpressing S312A IRS-1 (H4 S312A IRS-1) were generated as described under “Experimental Procedures.” H4IIE cells stably expressing either wt or the mutant S312A IRS-1 were stimulated with 10 nM insulin for 18 h. Anti-FLAG immunoprecipitates and cell lysates were analyzed by SDS-PAGE followed by transfer to nitrocellulose and immunoblotting. 70 ± 2 and 68 ± 2% (n = 3) reductions in wt IRS-1 protein levels were detected in anti-FLAG immunoprecipitates and total cell lysates, respectively. In contrast, the degradation of S312A IRS-1 was greatly reduced, whether the IRS-1 was detected in anti-FLAG immunoprecipitates with anti-IRS-1 immunoblotting or in total cell lysates with anti-FLAG immunoblotting (Fig. 5, A and C). The specificity of the anti-Ser(P)\textsuperscript{312} antibody used in these studies was confirmed by the finding that the IRS-1 S312A mutant protein only weakly reacted with this antibody compared with wild type IRS-1 (Fig. 5A).

To test the possibility that there is a general defect in the insulin-stimulated protein degradation in the H4 S312A IRS-1-expressing cells, H4 wt and S312A IRS-1 cells were stimulated with 10 nM insulin for 18 h, and the extent of degradation of the endogenous IRS-2 was measured. No difference was detected in the insulin-stimulated degradation of IRS-2 in H4 wt and S312A IRS-1 cells (Fig. 5B), indicating that there was no general decrease in the ability of insulin to stimulate IRS degradation in these cells expressing the mutant IRS-1.

Further studies were performed on both the dose and time dependence of wt and S312A mutant IRS-1 degradation. As above, controls were performed for total cellular protein by measuring the total levels of p85 in all the lysates. Insulin stimulated a dose-dependent decrease in wt IRS-1 levels with an ED\textsubscript{50} value of ~1 nM. In contrast, the S312A IRS-1 required ~100 times as much insulin to induce a comparable amount of degradation (ED\textsubscript{50} = ~100 nM) (Fig. 6A). The time dependence of the insulin-stimulated decrease in IRS-1 levels was also impaired in the S312A mutant as compared with the wt IRS-1. The IRS-1 degradation shifted from having a t\textsubscript{1/2} value of ~4 h for the wt IRS-1 to a t\textsubscript{1/2} value of ~14 h with the mutant IRS-1 (Fig. 6B).

Degradation of IRS-1 Inhibits Insulin Signaling—To test whether IRS-1 degradation inhibits insulin action, H4 wt IRS-1 cells were stimulated with or without insulin for 15 min, after pretreatment with 5 nM insulin for 24 h to induce IRS-1 degradation. The cell lysates were analyzed by SDS-PAGE followed by transfer to nitrocellulose and immunoblotting.
**ERK1/2 in vivo activation** was assessed by immunoblotting with an anti-phospho-ERK1/2 antibody. No differences were detected in ERK1/2 protein levels in **H4 wt IRS-1 cells** pretreated with 5 nM insulin for 24 h (Fig. 7A). However, a 65 ± 4% (n = 3) reduction in insulin-stimulated ERK1/2 activation was detected in **H4 wt IRS-1 cells** pretreated with 5 nM insulin for 24 h (Fig. 7A). To determine whether insulin-stimulated ERK1/2 activation was impaired to a similar extent in cells expressing the S312A mutant that is resistant to insulin-induced degradation, H4IE cells stably expressing either wt or the mutant S312A IRS-1 were pretreated with 5 nM insulin for 24 h to stimulate IRS-1 degradation and then stimulated with various doses of insulin for 15 min. ERK1/2 was activated to a greater extent in H4IE cells expressing the mutant S312A IRS-1 compared with wt expressing cells (Fig. 7B). These results indicate that S312A mutant IRS-1 that is more resistant to insulin-induced degradation confers on these cells a greater insulin response after a 24-h insulin pretreatment than the wild type IRS-1.

**Modulation of Insulin-stimulated IRS-1 Degradation by the JNK Inhibitor SP600125**—To further characterize the signaling pathway leading to insulin-stimulated IRS-1 degradation and Ser312 phosphorylation, **H4 wt IRS-1 cells** were pretreated with the JNK inhibitor SP600125 (62) for 30 min and then stimulated with 10 nM insulin for 18 h. The cell lysates were analyzed by SDS-PAGE followed by transfer to nitrocellulose and immunoblotting. In contrast to the 61 ± 6% degradation induced by insulin in the absence of inhibitor, insulin only induced a 27 ± 11% degradation in the presence of 25 μM SP600125 (Fig. 8, A and B). When 10 μM SP600125 was present, the ability of insulin to stimulate IRS-1 degradation was unaffected. In these experiments, insulin stimulated a ~7-fold increase in Ser312 phosphorylation, that was reduced by 53 ± 4% with SP600125 pretreatment at 25 μM (Fig. 8). However, pretreatment with SP600125 at 10 μM did not block Ser312 phosphorylation induced after a 18-h treatment with insulin.
Modulation of Insulin-stimulated c-Jun Phosphorylation by the JNK Inhibitor SP600125 and by the PI3 Kinase/Akt/mTOR Pathway—To correlate the ability of the various inhibitors used to block insulin-stimulated IRS-1 degradation and inhibit JNK activation, H4 wt IRS-1 cells were pretreated with the inhibitors for 30 min and then stimulated with 100 nM insulin for 15 min. The cells were lysed, and the total cell lysate or anti-FLAG immunoprecipitates (IP) were separated on 6% SDS-PAGE gels, transferred to nitrocellulose, and immunoblotted (IB). Two independent experiments were performed. Representative immunoblots, with anti-IRS-1 and anti-Ser(P)312 IRS-1 from anti-FLAG immunoprecipitates or with anti-p85 antibodies from total cell lysates, are shown. A, dose dependence of IRS-1 degradation. B, time course of insulin-stimulated IRS-1 degradation.

**DISCUSSION**

Numerous studies have demonstrated that Ser/Thr phosphorylation of IRS-1 inhibits insulin receptor-catalyzed IRS-1 tyrosine phosphorylation and the subsequent downstream signaling actions of insulin (24, 25, 63). One of the specific Ser phosphorylation sites in IRS-1 that has been proposed to negatively modulate the insulin signal is Ser312 (numbered according to the human sequence). Prior studies have demonstrated that IRS-1 associates with and is phosphorylated by JNK in vivo on Ser312 (34). Using a yeast tri-hybrid assay, it was shown that an active JNK blocked the interaction between the insulin receptor and IRS-1, thus suggesting that Ser312 phosphorylation may interfere with IRS-1 PTB domain function (64). Phosphorylation of IRS-1 at Ser312 also mediates the inhibitory effect of tumor necrosis factor α on insulin signaling, although the kinase responsible for this phosphorylation was inhibited by the MEK inhibitor PD98059 and thus is presum-
Insulin also stimulates the phosphorylation of IRS-1 at Ser\textsuperscript{312} by JNK-dependent and -independent pathways (65, 66). It has also been suggested that the effect of free fatty acids on IRS-1 tyrosine phosphorylation and associated PI 3-kinase activity in rats is mediated by activation of PKC\textsuperscript{\theta} leading to IRS-1 Ser\textsuperscript{312} phosphorylation (67). Thus, phosphorylation of IRS-1 at Ser\textsuperscript{312} by a variety of kinases may represent a general mechanism of inhibiting the insulin signal pathway by uncoupling IRS-1 from the insulin receptor.

In the present studies we have used a recently described inhibitor of JNK, SP600125 (62). Treatment of cells with 10 \textmu M SP600125 completely blocked insulin-stimulated \textit{in vivo} c-Jun phosphorylation. However, even 25 \textmu M SP600125 only partially inhibited insulin induced Ser\textsuperscript{312} phosphorylation. Further, inhibitors of the PI 3-kinase/Akt/mTOR pathway that block insulin-stimulated IRS-1 degradation and Ser\textsuperscript{312} phosphorylation had no effect on insulin-stimulated \textit{in vivo} c-Jun phosphorylation. These results are most consistent with a

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**Fig. 7.** **Degradation of IRS-1 inhibits insulin signaling.** H4IE cells expressing FLAG-tagged wt human IRS-1 (H4 wt IRS-1) or a S312A mutant (H4 S312A IRS-1) IRS-1 were first pretreated with or without 5 nM insulin for 24 h at 37 °C and then stimulated with or without insulin for 15 min at 37 °C. The cells were lysed, and the total cell lysates were separated on 10% SDS-PAGE gels, transferred to nitrocellulose and immunoblotted (IB) with the indicated antibodies. Immunoreactive bands were directly quantitated using a Kodak Work station and Kodak 1D v3.5.3 software. A, representative immunoblots with pERK1/2 and total ERK1/2 antibodies from H4 wt IRS-1 cells are shown in the left panel, and the quantitation of the results (means ± S.E.) of three independent experiments are summarized in the right panel. B, representative immunoblots with pERK1/2 and total ERK1/2 antibodies from H4 wt IRS-1 and H4 S312A IRS-1 cells are shown in the upper panel, and the quantitation of the results (means ± S.E.) of three independent experiments are summarized in the lower panel.

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FIG. 8. Effect of a JNK inhibitor on the degradation and phosphorylation of IRS-1. H4IE cells expressing FLAG-tagged wt human IRS-1 were pretreated with or without 25 or 10 μM SP600125 (SP) or 10 μM UO126 (UO) for 30 min at 37 °C followed by stimulation with 10 nM insulin for 18 h at 37 °C. The cells were lysed, and the total cell lysates were separated on 6% SDS-PAGE gels, transferred to nitrocellulose, and immunoblotted (IB). Immunoreactive bands were directly quantitated in duplicate using a Kodak Work station and Kodak 1D v.3.5.3 software. A, effect of the inhibitor on IRS-1 degradation and phosphorylation. Representative immunoblots with anti-IRS-1, anti-Ser(P)312 IRS-1, and anti-p85 antibodies are shown. B, quantitation of the effects of the inhibitors on the IRS-1 levels. IRS-1 protein levels are expressed as percentages of the nontreated control cells and are the means ± S.E. of three independent experiments. *, p < 0.05; **, p < 0.005 versus insulin-stimulated controls. C, quantitation of the effects of the inhibitors on IRS-1 Ser312 phosphorylation. Ser312 phosphorylation was quantitated, normalized for the amounts of IRS-1 present, and expressed as a percent of the insulin-stimulated control cells. The results shown are the means ± S.E. of three independent experiments. *, p < 0.05; **, p < 0.005 versus insulin-stimulated controls.
kinase other than JNK being responsible for the insulin-stimulated Ser312 phosphorylation of IRS-1. In contrast to our results in rat hepatoma cells, Lee et al. (66) recently reported that the PI 3-kinase inhibitor LY294002 blocked insulin-stimulated JNK activation and c-Jun phosphorylation in 32D cells overexpressing the insulin receptor. The finding that the JNK inhib-

Fig. 9. Effect of JNK, PI 3-kinase, and mTOR inhibitors on c-Jun and IRS-1 phosphorylation. H4IIE cells expressing FLAG-tagged wt IRS-1 were pretreated with or without 25 or 10 μM SP600125 (SP), 30 μM LY294002 (LY), 20 μM rapamycin (Rap), or 10 μM U0126 (UO) for 30 min at 37 °C followed by stimulation with 100 nM insulin for 15 min at 37 °C. The cells were lysed with sample buffer, and the total cell lysates were separated on 6% SDS-PAGE gels, transferred to nitrocellulose, and immunoblotted (IB). The immunoreactive bands were directly quantitated in duplicate using a Kodak Work station and Kodak 1D v.3.5.3 software. A, effect of JNK and MEK1/2 inhibitors on c-Jun phosphorylation. A representative immunoblot with anti-Ser(P)63 c-Jun is shown in the left panel, and the quantitation of the results (means ± S.E.) of three independent experiments are summarized in the right panel. B, effect on IRS-1 Ser312 and Ser616 phosphorylation. Representative immunoblots with anti-IRS-1, anti-Ser(P)312 IRS-1 and anti-Ser(P)616 IRS-1 are shown in the left panel, and the results (means ± S.E.) of three independent experiments are summarized in the right panel. C, effect of PI 3-kinase and mTOR inhibitors on c-Jun phosphorylation. A representative immunoblot with anti-Ser(P)63 c-Jun is shown in the left panel, and the quantitation of the results (means ± S.E.) of three independent experiments are summarized in the right panel.
itor partially blocks Ser\(^{616}\) phosphorylation of IRS-1 suggests that this inhibitor is not specific for JNK. Another recent report also concluded that this inhibitor is not specific for JNK (68). In the original report on this inhibitor (62), it was suggested that SP600125 may have activity toward several mitogen-activated protein kinase kinase isozymes and Akt based on in vitro screening and the partial inhibition of phospho-p38 and ATF in Jurkat T cells. In any case, our results indicate that JNK is unlikely to be the kinase phosphorylating IRS-1 Ser\(^{312}\) in response to insulin and are consistent with the hypothesis that multiple kinases mediate IRS-1 Ser\(^{312}\) phosphorylation. In agreement with this hypothesis is the recent evidence that IKK2 directly phosphorylated IRS-1 Ser\(^{312}\) (69).

A role for the PI 3-kinase cascade in the insulin-stimulated degradation of IRS-1 is supported by the finding that overexpression of a constitutively active form of the p110 subunit of PI 3-kinase is sufficient to induce IRS-1 degradation, whereas inhibition of PI 3-kinase by LY294002 inhibits insulin-stimulated IRS-1 degradation (43, 44, 46, 51). In H4IE cells stably expressing a wt IRS-1, inhibition of PI 3-kinase by LY294002 also completely blocked insulin-induced IRS-1 degradation. Further, inhibition of mTOR, a downstream effector of PI 3-kinase, by rapamycin also blocked insulin-stimulated IRS-1 degradation in these cells. The mechanism by which mTOR regulates the phosphorylation and subsequent degradation of IRS-1 is not known. It is possible that mTOR is associated with and regulates a phosphatase (70). It is also possible that mTOR directly phosphorylates IRS-1 Ser\(^{312}\), although phosphorylation at this Ser residue in IRS-1 was not previously identified (35).

The inhibitors used in the present work may act on targets in addition to their intended enzymes. Therefore, we sought to confirm the role of the PI 3-kinase cascade in the insulin-stimulated degradation of IRS-1. We first overexpressed PDK1, the upstream activator of the Akt pathway. We were able to show that overexpression of this kinase potentiated the ability of insulin to stimulate IRS-1 degradation. Even more important, we found that ES cells lacking PDK1 showed a dramatic decrease in the ability of insulin and insulin-like growth factor-I to stimulate IRS-1 degradation. These results therefore further document the hypothesized role of the PI 3-kinase cascade in the insulin-induced degradation of IRS-1 (42–44, 46). However, these studies are not consistent with a recent study in Chinese hamster ovary cells overexpressing the insulin receptor in which the mTOR inhibitor rapamycin was found not to block insulin induced IRS-1 degradation, possibly suggesting that there may be cell-specific pathways for IRS-1. To further clarify the steps whereby activation of the PI 3-kinase pathway leads to the degradation of IRS-1, we have examined the role of specific IRS-1 phosphorylation sites in this process. We found that inhibitors of the PI 3-kinase/Akt/mTOR pathway block insulin-stimulated IRS-1 degradation as well as inhibit Ser\(^{312}\) phosphorylation of IRS-1. We therefore produced an IRS-1 mutant in which Ser\(^{312}\) was changed to Ala and examined the ability of insulin to stimulate the degradation of this molecule. We found that this mutant IRS-1 was resistant to insulin-stimulated IRS-1 degradation. In contrast, a mutant IRS-1 in which Ser\(^{616}\) was changed to Ala exhibited a comparable insulin-stimulated degradation as the wt IRS-1. We next sought to investigate insulin action in H4IE cells in which IRS-1 was degraded.

Our results strongly suggest a novel dual mechanism for the inhibition of the insulin signal, whereby Ser\(^{312}\) phosphorylation uncouples IRS-1 from the insulin receptor as well as regulating its degradation. However, the finding that the IRS-1 mutant was degraded at high concentrations of insulin during a long term stimulation suggests that Ser\(^{312}\) phosphorylation is not the sole determinant for degradation.

The mechanism by which Ser\(^{312}\) phosphorylation promotes degradation is not known. It is possible that Ser\(^{312}\) phosphorylation promotes ubiquitination of IRS-1. However, the ability of insulin to stimulate ubiquitination of IRS-1 has not been consistently observed (47, 55). An alternative possibility for the mechanism by which Ser\(^{312}\) phosphorylation promotes degradation is that when phosphorylated, Ser\(^{312}\) facilitates the subcellular redistribution of IRS-1 from the low density microsomal or high speed pellet fraction to the cytosol. Such a redistribution would occur, for example, if the phosphorylation of Ser\(^{312}\) interferes with PTB function of the IRS-1 molecule (46). In support of such a model is the finding that rapamycin blocks the insulin-stimulated redistribution of IRS-1 from the low density microsomal fraction/high speed pellet to the cytosol (46). In the present studies rapamycin was also found to inhibit Ser\(^{312}\) phosphorylation. It is possible that these results are linked; that is, the phosphorylation of Ser\(^{312}\) may participate in the insulin-stimulated redistribution of IRS-1, which could subsequently facilitate IRS-1 degradation.

In conclusion, we have found that inhibition of IRS-1 degradation by inhibitors of the PI 3-kinase/Akt/mTOR pathway correlates with inhibition of IRS-1 Ser\(^{312}\) phosphorylation. Direct evidence for a participation of Ser\(^{312}\) phosphorylation in insulin-stimulated IRS-1 degradation was obtained because Ser\(^{312}\) mutant IRS-1 was found to be resistant to insulin-stimulated degradation. This study therefore provides the first evidence that a specific Ser/Thr phosphorylation site can modulate IRS-1 degradation.

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