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Nutrient-dependent and Insulin-stimulated Phosphorylation of Insulin Receptor Substrate-1 on Serine 302 Correlates with Increased Insulin Signaling*

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Ser/Thr phosphorylation of insulin receptor substrate IRS-1 regulates insulin signaling, but the relevant phosphorylated residues and their potential functions during insulin-stimulated signal transduction are difficult to resolve. We used a sequence-specific polyclonal antibody directed against phosphorylated Ser^{302} to study IRS-1-mediated signaling during insulin and insulin-like growth factor IGF-I stimulation. Insulin or IGF-I stimulated phosphorylation of Ser^{302} in various cell backgrounds and in murine muscle. Wortmannin or rapamycin inhibited Ser^{302} phosphorylation, and amino acids or glucose stimulated Ser^{302} phosphorylation, suggesting a role for the mTOR cascade. The Ser^{302} kinase associates with IRS-1 during immunoprecipitation, but its identity is unknown. The NH_2-terminal c-Jun kinase did not phosphorylate Ser^{302}. Replacing Ser^{302} with alanine significantly reduced insulin-stimulated tyrosine phosphorylation of IRS-1 and p85 binding and reduced insulin-stimulated phosphorylation of $p70^{S6K}$, ribosomal S6 protein, and 4E-BP1; however, this mutation had no effect on insulin-stimulated Akt or glycogen synthase kinase 3β phosphorylation. Replacing Ser^{302} with alanine reduced insulin/IGF-I-stimulated DNA synthesis. We conclude that Ser^{302} phosphorylation integrates nutrient availability with insulin/IGF-I signaling to promote mitogenesis and cell growth.

Many insulin signals are produced through tyrosine phosphorylation of the insulin receptor substrate (IRS)[1]-proteins or other scaffold proteins, including Shc, Cbl, APS, and SH2B, Gab1/2, and Dock1/2 (1). Although the role of each of these substrates merits attention, work with transgenic mice suggests that many insulin responses, especially those that are associated with somatic growth and carbohydrate metabolism, are mediated through IRS-1 or IRS-2 (2–4). IRS-proteins are composed of multiple interaction domains and phosphorylation motifs, but they appear to lack intrinsic catalytic activities (5). All IRS-proteins contain an NH_2-terminal pleckstrin homology domain followed by a phosphotyrosine binding domain. These domains couple the IRS-protein to the activated receptors for insulin, IGF-I, IL-4 and others (6). Multiple tyrosine phosphorylation sites in the COOH terminus recruit various enzymes (PI3K, SHP2, or fyn) or adapter proteins (Grb-2, nck, crk, shb, and others) that promote carbohydrate and lipid metabolism, cell growth and survival, and the function of specialized neuroendocrine tissues that promote nutrient homeostasis and female fertility (5, 7).

Insulin plays a pivotal role in metabolic regulation because it promotes nutrient influx and storage in muscle and adipocytes and inhibits hepatic gluconeogenesis. Although insulin is the principal activator, heterologous signals reflecting nutrient availability or physiological stress modulate insulin signaling. Dysregulation of these heterologous signals contributes to peripheral insulin resistance, metabolic dysregulation, and diabetes (2). Several mechanisms have been proposed to explain heterologous regulation of the insulin signaling cascade, including cytokine-induced serine phosphorylation or degradation of the IRS-proteins, or direct inhibition of the insulin receptor. IRS-1 contains more than 20 potential serine phosphorylation sites in amino acid sequence motifs that are recognized by various kinases, including protein kinase A, protein kinase B/Akt, protein kinase C, ERK1/2, Rho kinase and mTOR (8–10). Many studies suggest that serine phosphorylation causes insulin resistance by blocking the interaction between IRS-1 and the activated insulin receptor, inhibiting the phosphorylation of specific tyrosine residues, or promoting IRS-1 degradation (11–13). Previous work from our laboratory shows that JNK-mediated phosphorylation of Ser^{307} inhibits coupling of IRS-1 to the activated insulin receptor, which reduces insulin-stimulated tyrosine phosphorylation and insulin signaling (14, 15). By contrast, here we show that an adjacent residue, Ser^{302}, is required for insulin-stimulated tyrosine phosphorylation of IRS-1 and that its phosphorylation might link insulin signaling to nutrient availability.

MATERIALS AND METHODS

Antibodies and Reagents—Phosphospecific Ser^{302} antibodies were made by injecting rabbits with a synthetic peptide designed to contain phosphorylated Ser^{302} (Boston Biomolecule). Anti-phosphotyrosine antibody was purchased from Transduction Laboratories. Antibodies against IRS-1 and p85 have been described (16). Antibodies against phosphotyrosine, phospho-Akt, Akt, phospho-$p70^{S6K}$, $p70^{S6K}$, phospho-ribosomal S6, ribosomal S6, phospho-4E-BP-1, phospho-GSK-3β and GSK-3 were purchased from Cell Signaling Technology. JNK antibodies were from Santa Cruz. Monoclonal antibodies against full-length IRS-1 were made in the laboratory. All inhibitors, cytokines, and growth factors were purchased from Calbiochem. A BrdUrd cell proliferation

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[1] The abbreviations used are: IRS, insulin receptor substrate; $A302^{IRS-1}$, mutant IRS-1 protein containing an alanine substitution for Ser^{302}; BrdUrd, bromodeoxyuridine; CHO, Chinese hamster ovary; DMEM, Dulbecco's modified Eagle's medium; DPBS, Dulbecco's phosphate-buffered saline; ERK, extracellular signal-regulated kinase; glycogen synthase kinase; IGF, insulin-like growth factor; IL, interleukin; JNK, c-Jun NH_2-terminal kinase; MEF, mouse embryonic fibroblast(s); PI3K, phosphatidylinositol 3-kinase.

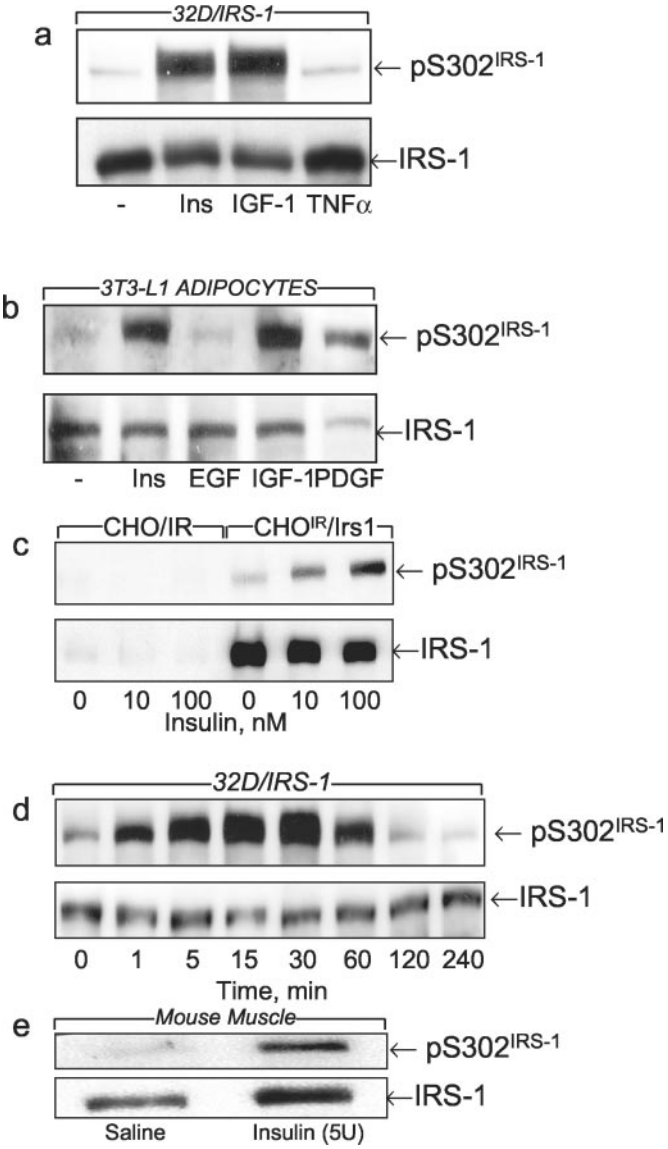

FIG. 1. **Insulin/IGF-I stimulates Ser^{302} phosphorylation.** *a*, 32D/IRS-1 cells were starved for 4 h in RPMI medium deprived of both serum and IL-3. The cells were subsequently treated with 100 nM insulin (*Ins*), 50 ng/ml IGF-I for 15 min, and 25 ng/ml tumor necrosis factor-α (*TNF*α) for 4 h. Whole cell lysates were resolved by SDS-PAGE and immunoblotted with α-pS^{302} and IRS-1 antibodies. *b*, murine 3T3-L1 adipocytes were starved overnight and treated with 100 nM insulin, 100 nM epidermal growth factor (*EGF*), 50 nM IGF-I, 10 nM platelet-derived growth factor (*PDGF*) for 30 min. Lysates were immunoprecipitated with IRS-1 monoclonal antibody for 3 h at 4 °C. Immunoprecipitates were resolved by SDS-PAGE and immunoblotted with α-pS^{302} and IRS-1 antibodies. *c*, CHO^{IR}/IRS-1 cells were starved in plain Ham's F-12 medium for 12 h and treated as in *a*. *d*, 32D/IRS-1 cells were starved for 4 h and treated with 100 nM insulin for the indicated times. Lysates were probed with α-pS^{302} and IRS-1 antibodies. *e*, muscle samples were prepared as described in Ref. 20. IRS-1 immunoprecipitates were blotted with α-pS^{302} and IRS-1 antibodies.

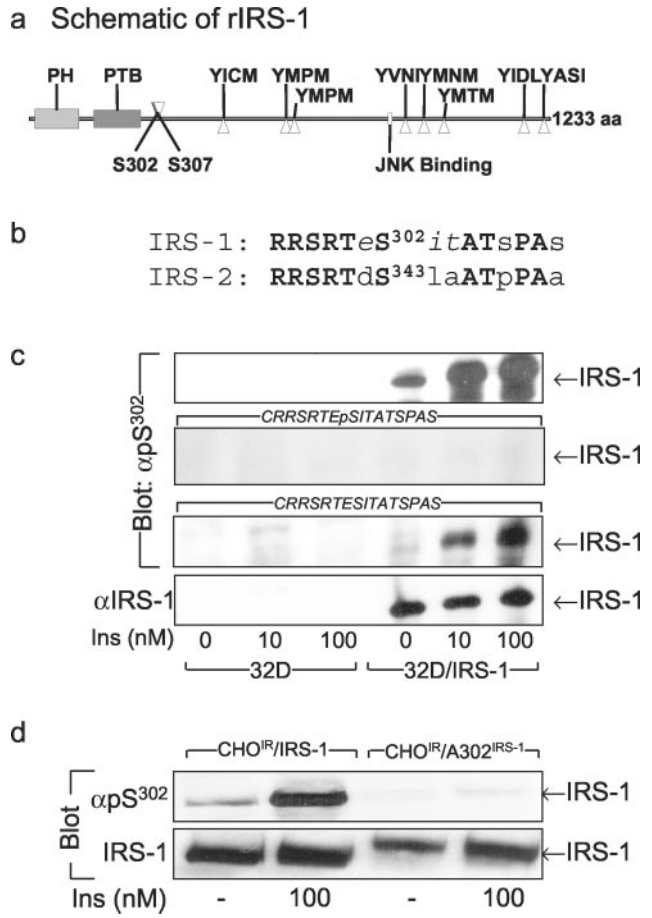

FIG. 2. **Specificity of the pS^{302} antibody.** *a*, schematic of rat IRS-1 illustrating the positions of Ser^{302} and YMXM motifs. *b*, comparison of peptide sequences surrounding Ser^{302} in IRS-1 and its corresponding serine residue (Ser^{343}) in mouse IRS-2. *c*, lysates from 32D and 32D/IRS-1 cells treated with 10 or 100 nM insulin (*Ins*) were immunoblotted with α-pS^{302} antibody (*top panel*), with a (1:1) mixture of the pS^{302} antibody and the phosphopeptide antigen (10 μM) it was raised against (*second panel* from *top*), with a (1:1) mixture of the pS^{302} antibody and the unphosphorylated version of the peptide antigen (10 μM) it was raised against (*third panel* from *top*), and with αIRS-1 (*bottom panel*). *d*, rat IRS-1 cDNA constructs containing wild type IRS-1 and IRS-1 with a mutation of Ser^{302} to Ala were transiently transfected into CHO/IR cells. CHO^{IR}/IRS-1 and CHO^{IR}/A302^{IRS-1} cells were starved overnight and treated with 100 nM insulin for 15 min. Lysates were immunoblotted with α-pS^{302} and IRS-1 antibodies. These results are representative of two independent experiments.

kit was purchased from Oncogene Research Products. Dulbecco's phosphate-buffered saline (DPBS), 50× amino acid solution, and dextrose were from Invitrogen. Mouse embryonic fibroblasts (MEFs) were from Roger Davis.

Mutagenesis—IRS-1 point mutants for Ser^{302} to Ala were generated using the Stratagene QuikChange XL site-directed mutagenesis method.

Cell Culture—IL-3-dependent 32D mouse myeloid cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, 5% WEHI (IL-3), and 5 mM histidinol. 32D express a few endogenous insulin receptors but no endogenous IRS-proteins. Overexpression of IRS-1 in 32D cells reconstitutes many aspects of the insulin signaling pathway (17, 18). These cells were starved for at least 4 h before treatment with insulin/IGF-I. All inhibitors were added 30 min before insulin or growth factor treatment at 37 °C. 32D/IRS-1 and 32D/A302^{IRS-1} (mutant IRS-1 protein containing an alanine substitution for Ser^{302}) were generated by electroporation and selected in histidinol as described previously (19). Chinese hamster ovary (CHO) cells were maintained in Ham's F-12 medium supplemented with 10% fetal bovine serum. MEFs were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum. CHO and MEF cells were starved for at least 12 h before inhibitor or insulin treatment.

Cell Lysis, Immunoprecipitation, and Western Analysis—32D cells were starved for 4 h in serum-free medium and stimulated with insulin for the indicated times. Cells were lysed in 50 mM Tris, pH 7.4, containing 130 nM NaCl, 5 mM EDTA, 1% Nonidet P-40, 100 mM NaF, 50 mM β-glycerophosphate, 100 μM vanadate, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 10 μg/ml aprotinin. Immunoprecipitations were performed for 2 h at 4 °C followed by collection on protein A/G-Sepharose. Lysates and immunoprecipitates were resolved by SDS-PAGE and transferred to nitrocellulose. Proteins were detected by immunoblotting with specific antibodies. A similar protocol was used for CHO and MEF cells. Muscle samples from mice were prepared as in Ref. 20.

Amino Acid Withdrawal—CHO^{IR}/IRS-1 cells were cultured in Ham's

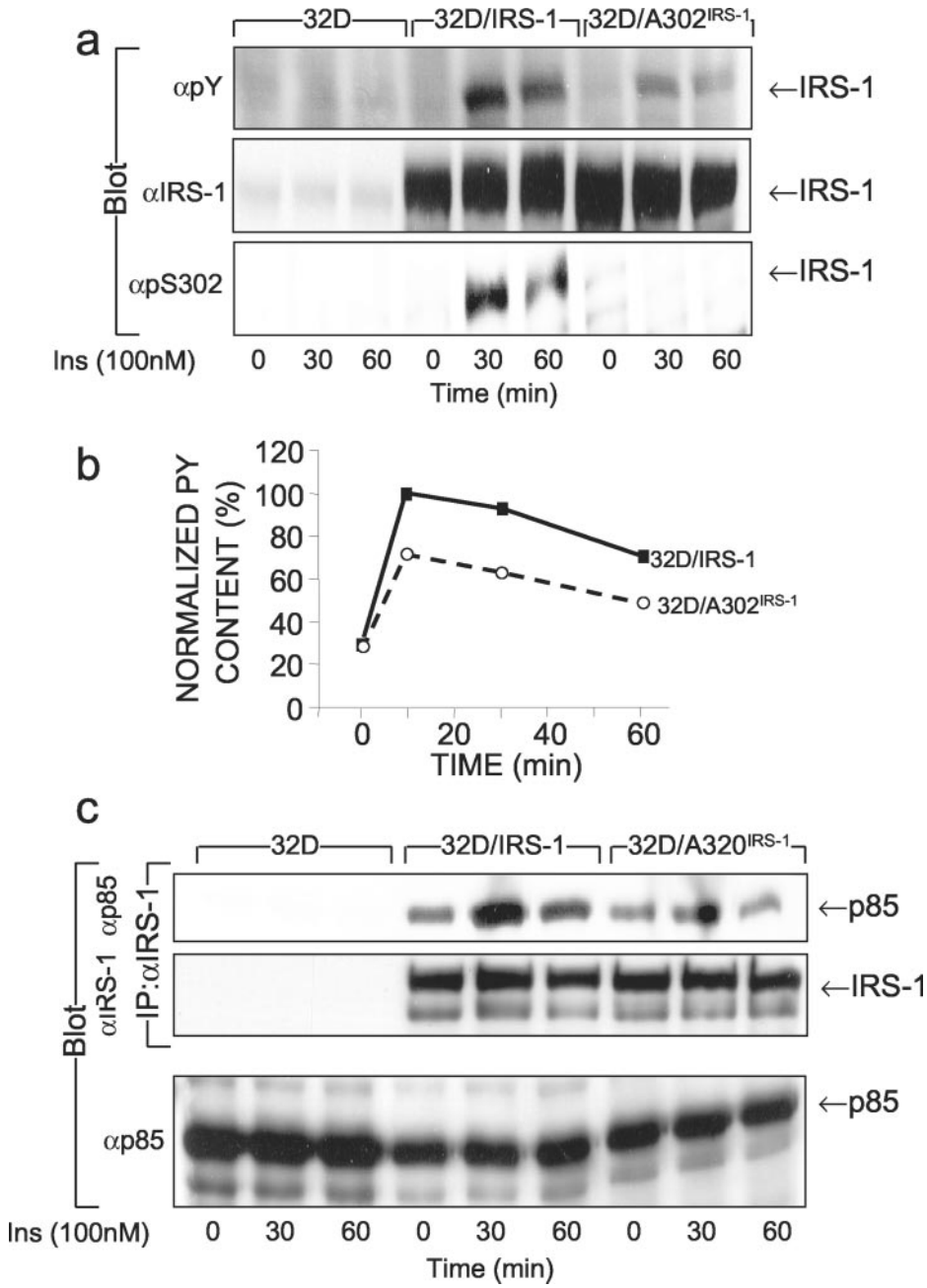

FIG. 3. **Mutation of Ser[302] to Ala reduced insulin/IGF-I-stimulated IRS-1 tyrosine phosphorylation.** *a*, 32D cells expressing rat IRS-1 or A302[IRS-1] or an empty vector were treated with 100 nM insulin (*Ins*) for the indicated times. IRS-1 immunoprecipitates were blotted with anti-phosphotyrosine (*αPY*), IRS-1, and α-pS[302] antibodies. *b*, 32D/IRS-1 and 32D/A302[IRS-1] cells were treated with 100 nM insulin for the indicated times, and lysates were blotted directly with α-PY-20 and α-IRS-1. The phospho-IRS-1 band was normalized to the corresponding IRS-1 band (total) using ImageQuant, and the results are reported as percentages of the maximum value obtained. These results are representative of three independent experiments using three different clones. Standard deviations are less than 5% in each case, and error bars are omitted for clarity. *c*, 32D, 32D/IRS-1, and 32D/A302[IRS-1] cells were treated with 100 nM insulin for the indicated times. IRS-1 immunoprecipitates were blotted with αp85 and IRS-1 antibodies. Lysates were immunoblotted with p85 antibody.

F-12 medium as described previously. For the starvation of amino acids, cells were starved for at least 12 h in serum-free medium. Cells were then washed twice with and incubated in the same buffer for 2 h. A 1× final concentration of amino acids or 5 mM of D-glucose (Glc) were added to the cells for 30 min followed by a 15-min treatment with insulin (21).

Cell Proliferation Assay—Insulin/IGF-I-stimulated proliferation of 32D cells was assayed according to the company's manual (Oncogene Research Products). Briefly, 32D cells in log phase growth were washed twice in phosphate-buffered saline and counted by the trypan blue exclusion method using a hematocytometer. 100 μl of cells at a density of 1×10^4 cells/ml were seeded into a 96-well culture dish. Cells were allowed to grow for 24 h. BrdUrd label was added to the cells for 6 h at 37 °C. BrdUrd incorporation was measured by enzyme-linked immunosorbent assay using a dual wavelength spectrometric plate reader ($A_{450-540\ nm}$). As controls, we included a blank containing only tissue culture medium and a background control containing only cells that were not labeled with BrdUrd. Each condition was done in triplicate, and the average absorbance values were used in plotting the results.

RESULTS

Insulin/IGF-I Stimulates Ser[302] Phosphorylation—To study serine phosphorylation of IRS-1, we raised sequence-specific polyclonal antibodies against 17-amino acid peptides containing putative serine phosphorylation sites in rat IRS-1. One of these antibodies was directed against pSer[302] and designated α-pS[302]. The α-pS[302] immunoblotted IRS-1 extracted from insulin or IGF-I-stimulated 32D/IRS-1 cells, 3T3-L1 adipocytes, and CHO[IR]/IRS-1 cells. Platelet-derived growth factor had a weak effect in 3T3-L1 adipocytes, and epidermal growth factor and tumor necrosis factor-α did not promote Ser[302] phosphorylation (Fig. 1, *a–c*). Ser[302] phosphorylation was detected 1 min after insulin stimulation of 32D/IRS-1 cells, reached a maximum within 30 min, but was barely detected after 2 h (Fig. 1*d*). Moreover, Ser[302] was phosphorylated in IRS-1 isolated from muscles of insulin-stimulated mice, suggesting that Ser[302] phosphorylation might be physiologically relevant (Fig. 1*e*).

We conducted peptide competition and site-directed mutagenesis experiments to establish the specificity of the α-pS[302]. The phosphopeptide used to produce α-pS[302] (Fig. 2, *a* and *b*) completely prevented immunoblotting of IRS-1 from

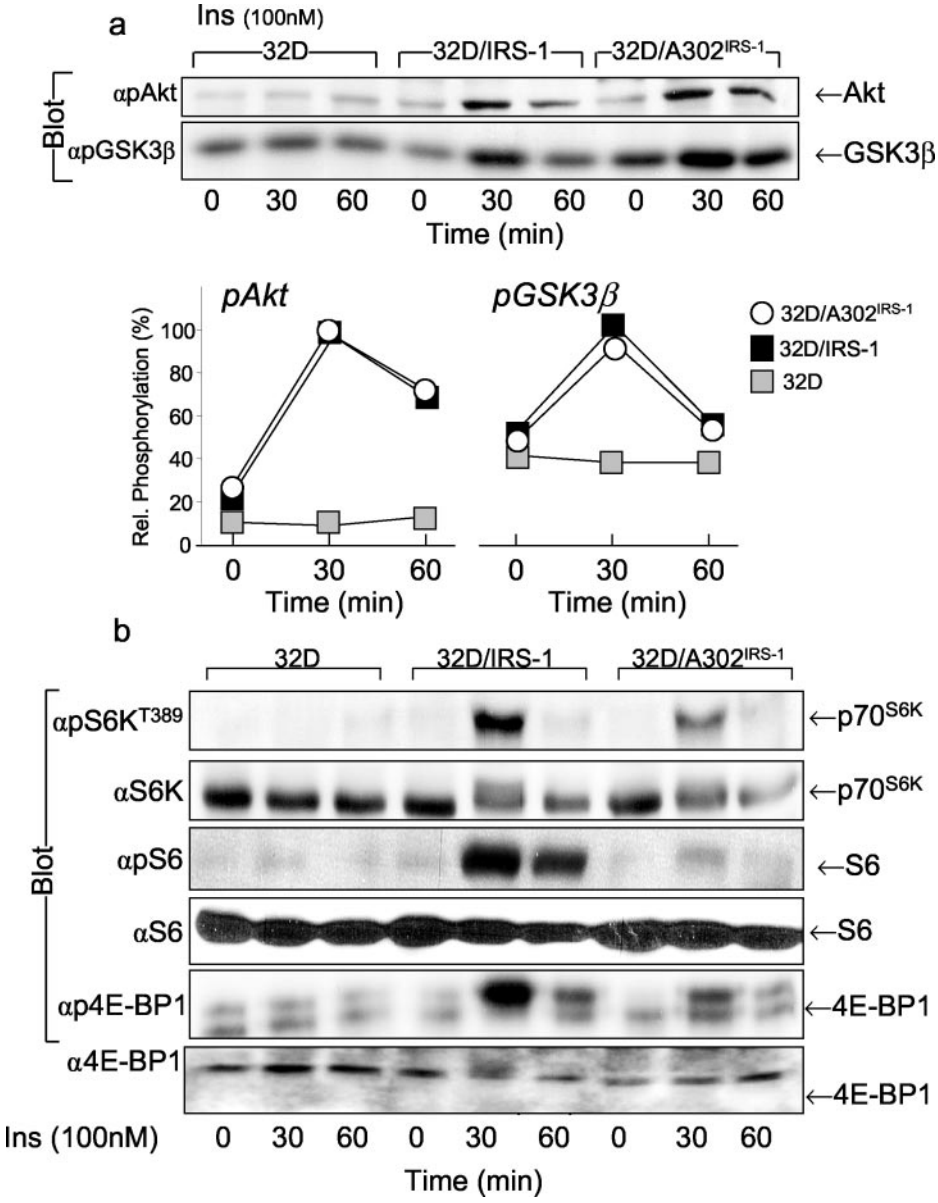

FIG. 4. **Effect of Ser^{302} → Ala mutation on insulin signaling.** 32D, 32D/IRS-1, and 32D/A302^{IRS-1} cells were treated with 100 nM insulin (*Ins*) for the indicated times. *a*, lysates were immunoblotted directly with α-phospho-Akt ^{S473} and α-phospho-GSK-3β^{Ser9} antibodies. Quantification of phospho-Akt and phospho-GSK-3β immunoblots was performed using ImageQuant. Basal and insulin-stimulated Akt activity was estimated for 32D, 32D/IRS-1, and 32D/A302^{IRS-1} cells by normalizing the total phospho-Akt signal to the corresponding total Akt signal for each band. Results represent the means of three independent experiments and are expressed as percentage of the maximum signal obtained in each experiment. A similar procedure was used for quantifying phospho-GSK-β. *b*, lysates were immunoblotted directly with α-phospho-p70^{Thr389}, α-p70^{S6K}, α-phospho-S6$^{Ser235/236}$ ribosomal protein, α-S6 ribosomal protein, α-phospho-4E-BP1$^{Thr\ 37}$, and α-4E-BP1.

insulin-stimulated 32D/IRS-1 cells (Fig. 2*c*). The unphosphorylated peptide at best weakly inhibited immunoblotting, suggesting that the phosphoserine residue was an important determinant of specificity (Fig. 2*c*). The α-pS^{302} did not immunoblot background proteins in the various cell extracts, did not immunoblot alkaline phosphatase-treated IRS-1 immunoprecipitates, and it did not immunoblot IRS-2, even though the corresponding serine residue (Ser^{343}) is phosphorylated (data not shown). Replacing Ser^{302} with Ala in IRS-1 completely abolished immunoblotting by α-pS^{302} (Fig. 2*d*). Based upon these results, we conclude that α-pS^{302} is specific for IRS-1 phosphorylated on Ser^{302}.

Mutation of Ser^{302} to Ala Impairs Insulin Signaling—Serine phosphorylation is generally thought to inhibit IRS-1 function by decreasing insulin-stimulated tyrosine phosphorylation or promoting IRS-1 degradation through a proteasome-mediated degradation pathway (22). For example, JNK-mediated phosphorylation of Ser^{307} in IRS-1 inhibits insulin signaling; replacing Ser^{307} with alanine increases insulin-stimulated IRS-1 tyrosine phosphorylation and downstream signaling (15). To establish the effect of Ser^{302} on IRS-1 function, we generated 32D cells expressing wild type IRS-1 or a mutant IRS-1 protein containing an alanine substitution for Ser^{302} (A302^{IRS-1}). Stable 32D cell clones expressing equal amounts of IRS-1 or A302^{IRS-1} were identified by immunoblotting. Insulin strongly stimulated IRS-1 tyrosine and Ser^{302} phosphorylation in 32D/IRS-1 cells, whereas tyrosine phosphorylation was reduced significantly in A302^{IRS-1} (Fig. 3, *a* and *b*). Consistent with reduced tyrosine phosphorylation, A302^{IRS-1} associated weakly with p85 during insulin stimulation (Fig. 3*c*). We conclude that Ser^{302} promotes IRS-1 function during insulin stimulation.

IRS-1 Signaling Is Regulated Differentially through Ser^{302}—The IRS-1/PI3K cascade regulates various downstream signals during insulin/IGF-I stimulation, including the activation of Akt/GSK-3β, p70^{S6K}/S6 and mTOR/4E-BP1 cascades. As shown previously, IRS-1 was required for insulin-stimulated phosphorylation of Akt in 32D cells, which is consistent with the binding and activation of PI3K by IRS-1 during insulin stimulation (Fig. 4*a*). However, insulin stimulated normally Akt phosphorylation in 32D/A302^{IRS-1} cells, even though tyrosine phosphorylation of A302^{IRS-1} and p85 recruitment was significantly reduced during insulin stimulation (Fig. 4*a*). Akt phosphorylates GSK-3β on Ser^{9}, which can be detected with a phosphospecific antibody (23). Phosphorylation of GSK-3β was stimulated equally by insulin in 32D/IRS-1 and 32D/A302^{IRS-1} cells, suggesting that replacing Ser^{302} with alanine did not

impair IRS-1-mediated activation of the Akt/GSK-3β cascade during insulin stimulation (Fig. 4*a*).

The activation of p70^{S6K} was investigated during insulin stimulation by immunoblotting with a phosphospecific antibody against pThr^{389}. As shown previously, insulin failed to stimulate p70^{S6K} phosphorylation in 32D cells, whereas it was strongly phosphorylated in 32D/IRS-1 cells, especially after 30 min of insulin stimulation (Fig. 4*b*). The ribosomal protein S6 and the regulatory protein 4E-BP1 are substrates of p70^{S6K} and mTOR, respectively, and both proteins were phosphorylated during insulin stimulation of 32D/IRS-1 cells (Fig. 4*b*). However, insulin weakly stimulated p70^{S6K} phosphorylation in 32D/A302^{IRS-1} cells, and the phosphorylation of S6 and 4E-BP1 was barely detected (Fig. 4*b*). Thus, Ser^{302} was essential for the mTOR/p70^{S6K} branch of the IRS-1 signaling cascade.

A Ser^{302} Kinase Associates with IRS-1—Specific association between a kinase and its substrates, followed by the recognition of phosphorylation sites by the associated kinase, determines the specificity of protein phosphorylation. To determine whether the Ser^{302} kinase associates with IRS-1, specific immunoprecipitates containing IRS-1 were incubated without or with 500 μM ATP. Before addition of ATP, Ser^{302} was phosphorylated as observed previously; however, Ser^{302} phosphorylation increased 2-fold after incubation with ATP (Fig. 5*a*) Thus, a Ser^{302} kinase associated with IRS-1 during immunoprecipitation.

Previous studies show that JNK phosphorylates Ser^{307} on rat IRS-1 (Ser^{312} in human IRS-1), which inhibits insulin signaling (24). Because Ser^{302} is close to Ser^{307}, we tested whether JNK might phosphorylate Ser^{302}. We conducted this experiment with wild type MEFs or JNK-deficient MEFs obtained from JNK-1−/−::JNK-2−/− mice (MEFs do not express JNK-3). Our previous work showed that Ser^{307} phosphorylation was decreased more than 50% in JNK1−/−::JNK2−/− MEFs (15). However, insulin or IGF-I stimulated Ser^{302} phosphorylation to similar levels in both wild type and JNK-1−/−::JNK-2−/− MEFs (Fig. 5*b*). Because recombinant JNK-1 did not phosphorylate Ser^{302} *in vitro* (data not shown), we conclude that insulin/IGF-I-stimulated phosphorylation of Ser^{302} in IRS-1 was not mediated by JNK. These results reveal that adjacent sites in IRS-1 are phosphorylated through distinct associated kinases.

Ser^{302} Phosphorylation Is Downstream of mTOR—We used classical inhibitors and amino acid starvation to determine whether the mTOR cascade might mediate Ser^{302} phosphorylation in IRS-1. Wortmannin or LY294002 (PI3K cascade), or rapamycin (mTOR cascade) inhibited insulin-stimulated Ser^{302} phosphorylation in MEFs, whereas PD98059 (mitogen-activated protein kinase cascade) had no inhibitory effect; these inhibitors had the expected effects on p70^{S6K}, ERK1/2, and Akt phosphorylation (Fig. 5*c*). Similar results were obtained with 32D, CHO cells, and 3T3-L1 adipocytes (data not shown). Thus, mTOR-regulated pathways promote insulin-stimulated Ser^{302} phosphorylation.

Previous results showed that activation of mTOR by mitogens or insulin is modulated by nutrient availability, including amino acids or glucose (25, 26). We investigated the effect of amino acid and glucose starvation on Ser^{302} phosphorylation to establish further whether an mTOR-mediated pathway is involved. Incubation of CHO^{IR}/IRS-1 cells in glucose and amino acid-free medium (DPBS) reduced basal and insulin-stimulated Ser^{302} phosphorylation (Fig. 6, *a* and *b*). Consistent with a role for the mTOR cascade, the addition of amino acids or glucose restored basal and insulin-stimulated Ser^{302} phosphorylation (Fig. 6, *a* and *b*). Insulin-stimulated AKT phosphorylation was not reduced in glucose/amino acid-free medium, suggesting that the early steps of insulin signaling were not inhibited, and sufficient ATP exists in the starved cells to mediate kinase activities (Fig. 6*b*). Rapamycin or LY294002 inhibited amino acid-stimulated phosphorylation of Ser^{302}, whereas PD98059 had no effect, which was consistent with a role for the mTOR cascade during nutrient and insulin-stimulated Ser^{302} phosphorylation (Fig. 6*c*).

Although our results suggest that kinases in the mTOR cascade promote Ser^{302} phosphorylation, mTOR itself is probably not involved. We treated cells with a relatively high concentration of okadaic acid, which inhibits protein phosphatase 2A and other related phosphatases (27). As expected, okadaic acid increased Ser^{302} phosphorylation; however, the stimula-

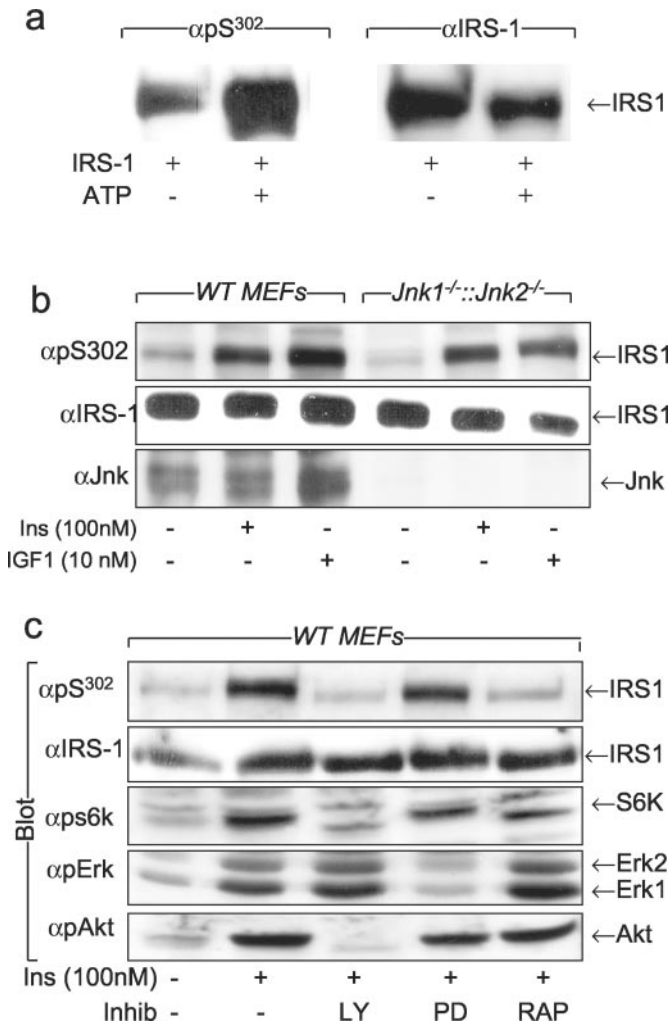

FIG. 5. **Insulin-stimulated Ser^{302} phosphorylation occurs downstream of mTOR.** *a*, IRS-1 was immunopurified from serum-starved CHO^{IR}/IRS-1 cells using an IRS-1 monoclonal antibody. Purified IRS-1 was incubated with either buffer A alone (50 mM Hepes, pH 7.4, 0.5 M NaCl, 1 mM dithiothreitol, 20 mM β-glycerophosphate, 1 mM phenylmethylsulfonyl fluoride) or with buffer A supplemented with 500 μM ATP. The reaction was allowed to proceed at 30 °C for 30 min. Samples were resolved by SDS-PAGE and immunoblotted with α-pS^{302} and IRS-1 antibodies. *b*, wild type (*WT*) and JNK$^{-/-}$ MEFs were starved overnight in DMEM High and treated with 100 nM insulin or IGF-I for 10 min. IRS-1 was immunoprecipitated with α-IRS-1 monoclonal antibody and immunoblotted with α-pS^{302} and IRS-1 antibodies. Lysates were blotted directly with anti-SAPK antibodies. *c*, MEF cells were preincubated for 30 min with 20 μM LY294002 (*LY*), 50 μM PD98059 (*PD*), or 100 nM rapamycin (*RAP*) before stimulation with 100 nM insulin for an additional 10 min. IRS-1 immunoprecipitates were blotted with α-pS^{302} and α-IRS-1 antibodies, and lysates were immunoblotted directly with antibodies against phospho-p70S6K^{Thr389}, phospho-mitogen-activated protein kinase$^{T202/Y204}$, and phospho-Akt ^{S473}.

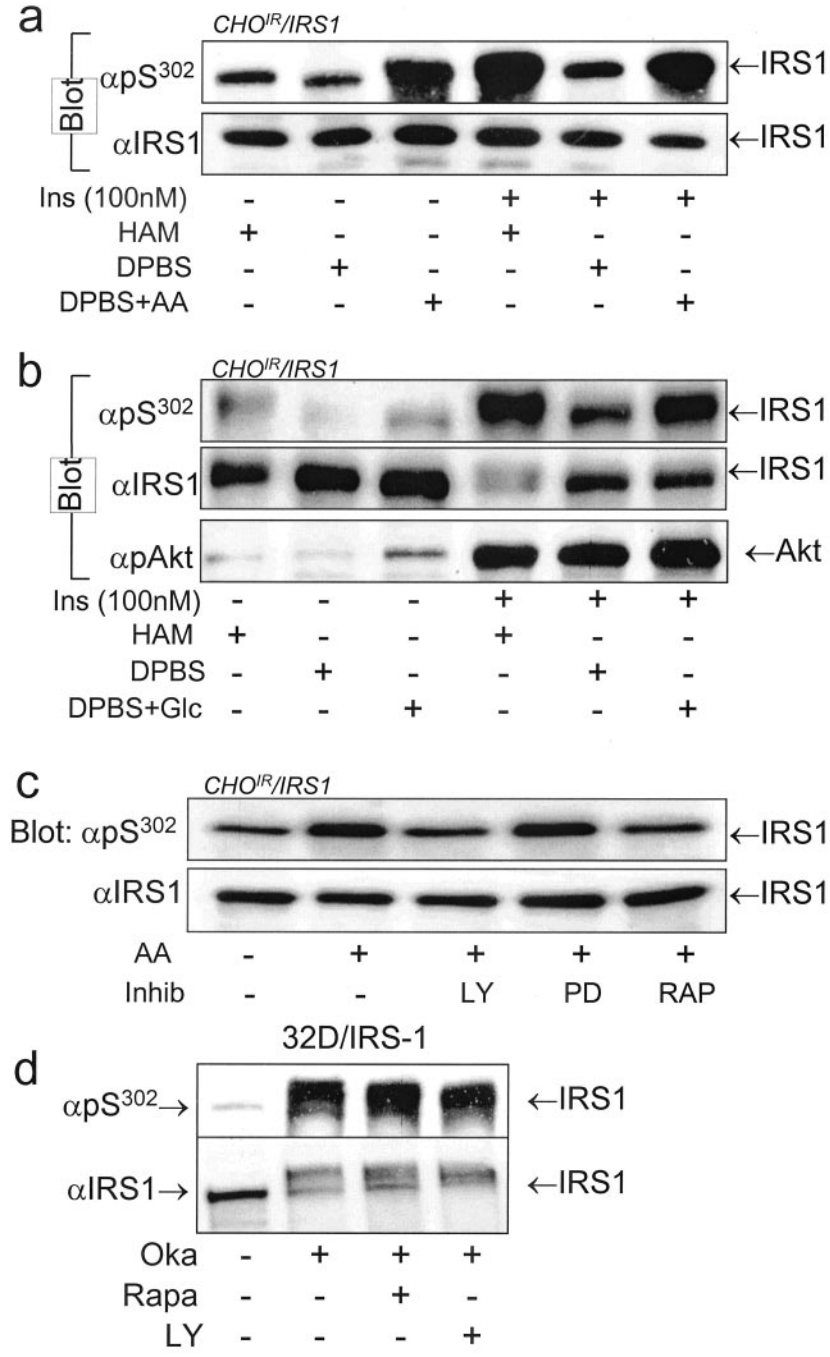

FIG. 6. **Nutrients regulate insulin stimulation of Ser^{302} phosphorylation.** *a*, CHO^{IR}/IRS-1 cells were starved for 20 h in Ham's F-12 medium and then incubated in DPBS for 2 h or left in the serum-free Ham's F-12 medium (HAM). A 1× amino acid solution (*AA*) was added for 30 min only to the cells in DPBS. Then 100 nM insulin was added to both the cells in DPBS and serum-free medium (HAM) for an additional 10 min. Lysates were immunoblotted with α-pS^{302} and IRS-1 antibodies. *b*, same as in *a* except that 5 mM dextrose (*Glc*) was added to DPBS instead of 1× amino acids. *c*, cells were starved for 20 h in Ham's F-12 serum-free medium and incubated for 2 h in DPBS. 20 μM LY294002 (*LY*), 50 μM PD98059 (*PD*), or 100 nM rapamycin (*RAP*) was added to the cells 30 min prior to the addition of 1× amino acids for 30 min. Lysates were immunoblotted with α-pS^{302} and IRS-1 antibodies. *d*, 32D/IRS-1 cells were starved for 4 h and treated with 100 nM rapamycin or 20 μM LY294002 for 30 min before adding 1 μM okadaic acid (*Oka*) for 1 h. Lysates were probed with αpS^{302} and IRS-1 antibodies.

tory effect of okadaic acid was not inhibited by either rapamycin or LY294002 (Fig. 6*d*). We conclude that mTOR is not a direct Ser^{302} kinase.

Ser^{302} Promotes Insulin-stimulated Mitogenesis—One of the main biological functions of insulin/IGF-I is to promote cell proliferation, and the activation of Akt, p70^{S6K}, and 4E-BP1 plays an important role (28–31). Because these signals are regulated differentially by A302^{IRS-1}, we compared insulin/IGF-I-stimulated DNA synthesis in 32D/IRS-1 and 32D/A302^{IRS-1} cells. As shown previously, insulin or IGF-I failed to promote DNA synthesis in wild type 32D cells; however, expression of IRS-1 mediated insulin/IGF-I-stimulated DNA synthesis (Fig. 7). Because insulin receptors are nearly absent in 32D cells, a high concentration of insulin was required to stimulate DNA synthesis in 32D/IRS-1 cells. However, insulin or IGF-I stimulation of DNA synthesis was significantly reduced in 32D/A302^{IRS-1} cells (Fig. 7).

DISCUSSION

Our results show that Ser^{302} in rat/mouse IRS-1 is phosphorylated rapidly during insulin stimulation. Although we have not identified the kinase, inhibitor experiments and short term glucose/amino acid starvation suggest that mTOR-regulated kinases are involved. TOR is thought to act as a sensor of ambient amino acid concentrations (32, 33). Insulin-stimulated Ser^{302} phosphorylation is reduced when glucose/amino acids are removed from the culture medium and restored to normal when these nutrients are provided. These results reveal a potential mechanism that coordinates IRS-1-mediated insulin action with nutrient availability.

Replacing Ser^{302} with alanine (A302^{IRS-1}) provides the strongest evidence that Ser^{302} is a cornerstone of IRS-1 function. A302^{IRS-1} is expressed normally in cells, but it is phosphorylated poorly on tyrosine residues and interacts weakly

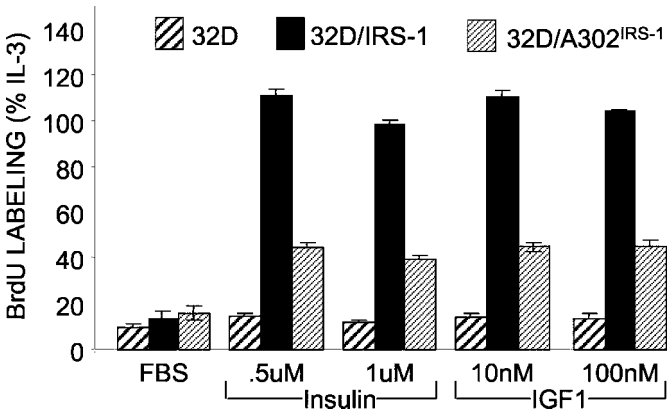

FIG. 7. **Ser^{302} phosphorylation participates in Insulin/IGF-I-stimulated proliferation of 32D cells.** BrdUrd cell proliferation assays were conducted according the manufacturer's manual (Oncogene Research Products). 32D, 32D/IRS-1, and 32D/A302^{IRS-1} cells were seeded at a density of 1×10^4 cells/ml and grown in a 96-well plate for 24 h. Cells were incubated with 10% fetal bovine serum (*FBS*), 0.5 μM and 1 μM insulin, 10 nM and 100 nM IGF-I, and 5% WEHI (IL-3). BrdUrd was added for 6 h, and absorbance was measured against a background control (see "Materials and Methods"). Insulin/IGF-1-stimulated cell proliferation was compared with the proliferation of cells incubated in IL-3-containing medium (WEHI). Each condition was done in triplicate and results are expressed as percentages of incorporation obtained with cells incubated with IL-3 (*% IL-3*). The results represent two independent experiments.

with p85 during insulin stimulation. Previous work from our laboratory and elsewhere establishes a close relation between p85 binding to IRS-1 and activation of the PI3K. This relation is especially clear in 32D and 32D^{IR} cells that lack endogenous IRS-proteins (19, 34, 35). Phospholipid products generated from PI3K activation activate various serine kinases, including Akt and certain atypical protein kinase C isoforms (36). Akt plays an important role in insulin action as it phosphorylates GSK-3β, the antiapoptotic protein BAD, the forkhead transcription factor Foxo1, and other targets (37, 38). In 32D or 32D^{IR} cells, expression of recombinant IRS-1 is a prerequisite for activation of Akt by insulin (19). However, expression of IRS-1 rather than its ability to activate PI3K might be the critical step in Akt activation in 32D cells. Here we show that A302^{IRS-1} barely binds to p85, whereas it mediates insulin-stimulated phosphorylation of Akt and the activation of the Akt/GSK-3β cascade normally. This result is consistent with early findings that a truncated IRS-1 protein composed of residues 1–309 failed to promote PI3K activity whereas activating Akt during insulin stimulation (19). Moreover, similar conclusions have emerged during that study of various models of insulin resistance, where PI3K is poorly activated whereas Akt is activated normally (39, 40).

Insulin/IGF signaling plays an important role in somatic growth, and disruption of IRS-1 results in mice that reach 50% or their expected size (41). Body growth depends upon mitogenesis and protein synthesis. Translational control by amino acid-dependent signaling appears to be integrated with the insulin stimulated PI3K signaling through the activation of TOR, the target of rapamycin (17, 26). Like Akt activation, the expression of IRS-1 is absolutely required for insulin stimulation of the p70^{S6K}/S6 pathway and inactivation of 4E-BP1; however, in this case the activation of PI3K is essential (17, 34). Unlike our results with the Akt/GSK-3β cascade, A302^{IRS-1} fails to mediate the insulin stimulation of the p70^{S6K}/S6 pathway and phosphorylation of 4E-BP1. The truncated IRS-1-protein composed of residues 1–309 also fails to mediate p70^{S6K} activation, consistent with its inability to bind p85 (19). Moreover, a mutant IRS-1 molecule lacking 18 tyrosine phosphorylation sites (F18^{IRS-1}) fails to bind p85 and activate PI3K and does not mediate insulin-stimulated phosphorylation of 4E-BP1 (17, 34). Thus, Ser^{302} is a specificity determinant in the IRS-1 branch of the insulin/IGF signaling cascades which can control various aspects of the signal.

Replacing Ser^{302} with alanine causes a significant inhibition of insulin-stimulated tyrosine phosphorylation of IRS-1. Because Ser^{302} has a positive role in IRS-1 tyrosine phosphorylation, its replacement by alanine fails to establish whether this residue is functionally important in the phosphorylated or unphosphorylated state. However, the finding that inhibition of Ser^{302} phosphorylation by short term amino acid/glucose starvation also correlates with a decrease in IRS-1 tyrosine phosphorylation, without inhibition of insulin receptor autophosphorylation or Akt phosphorylation, supports the conclusion that Ser^{302} phosphorylation might be important[2] (25). We do not know the mechanism by which Ser^{302} phosphorylation has this effect. Ser^{302} phosphorylation might stabilize the interaction between the phosphotyrosine binding domain of IRS-1 and the juxtamembrane region of the insulin receptor, which is expected to promote tyrosine phosphorylation. However, Ser^{302} phosphorylation might inhibit the recruitment of phosphotyrosine phosphatases or inhibit interactions with serine/threonine kinases that phosphorylate negative regulatory sites. Because the IRS-1-branch of the insulin/IGF signaling cascade plays an important role in protein synthesis, phosphorylation of Ser^{302} might serve to attenuate insulin action when nutrients are in short supply (17).

Regulation of IRS-protein levels and function is an important component of the insulin/IGF response in various tissues (43, 44). Several mechanisms controlling IRS-protein levels have been investigated, including regulation of IRS-2 gene expression by cAMP-responsive element-binding protein, or proteasome-mediated degradation of IRS-1 and IRS-2 (22, 45, 46). Regulation of IRS-protein function by serine/threonine phosphorylation mediated by heterologous kinases is also an important control mechanism (47). Most studies suggest that serine/threonine phosphorylation inhibits insulin-stimulated tyrosine phosphorylation (9, 48, 50). However, IRS-proteins contain more than 20 potential serine/threonine phosphorylation sites, and some of these sites are reported to promote tyrosine phosphorylation (51, 52). In addition to Ser^{302} reported here, Ser^{789} in IRS-1 is phosphorylated by the AMP kinase. Phosphorylation of Ser^{789} is reported to increase insulin signaling (52).

Our previous work focused on the phosphorylation of Ser^{307} in rat/mouse IRS-1 (Ser^{312} in human IRS-1), which is located near Ser^{302} (14, 15). Both Ser^{302} and Ser^{307} are phosphorylated during insulin stimulation. Replacing Ser^{307} with alanine increases insulin-stimulated tyrosine phosphorylation of IRS-1, suggesting that phosphorylation of Ser^{307} blocks insulin receptor tyrosine phosphorylation by inhibiting phosphotyrosine binding domain function (14). Phosphorylation of Ser^{307} inhibits insulin-stimulated tyrosine phosphorylation of IRS-1, which is reversed by mutation of Ser^{307} to alanine (15).

Both Ser^{302} and Ser^{307} are phosphorylated by kinases that associate with IRS-1 during immunoprecipitation. At least two kinases promote Ser^{307} phosphorylation, including the JNK, which associates with IRS-1 at a well defined COOH-terminal binding site (15). Mutation of the JNK binding site or disruption of JNK1/2 significantly inhibits insulin-stimulated Ser^{307} phosphorylation (15, 53). However, mutation of the JNK binding site in IRS-1 or disruption of JNK isoforms does not reduce insulin-stimulated Ser^{302} phosphorylation, suggesting that

[2] J. Giraud, R. L. Leshan, Y. H. Lee, and M. F. White, unpublished observations.

Ser^{302} is not a JNK phosphorylation site. These results reveal the striking site-specific phosphorylation that occurs in IRS-1.

The Ser^{302} kinase is unknown; however, inhibition of Ser^{302} phosphorylation by wortmannin or rapamycin, and by short term amino acid/glucose starvation, implicates the PI3K/mTOR cascade. However, Ser^{302} is probably not directly phosphorylated by mTOR, because the serine phosphatase inhibitor okadaic acid promotes Ser^{302} phosphorylation in a rapamycin-insensitive manner. Insulin signaling through mTOR has been shown to inhibit protein phosphatase 2A activity, which might contribute to Ser^{302} phosphorylation (42). However, inhibition of protein phosphatase 2A might be a nonspecific mechanism that would lead to increased phosphorylation of many serine residues in IRS-1.

The PI3K/mTOR/S6K pathway promotes cell growth and proliferation in cell-based experiment and in adult mouse tissues (30, 49). Deletion of p70^{S6K} or ribosomal protein S6 in embryonic stem cells or mice reduces the rate of cell proliferation (31). Consistent with those data, we observed a decrease in insulin/IGF-I-stimulated cell proliferation in 32D cells expressing the Ser^{302} to Ala mutation. The decrease in IRS-1 tyrosine phosphorylation and down-regulation of the PI3K/mTOR/S6K pathway is likely responsible for this defect in cell proliferation. Reduction in IRS-1 tyrosine phosphorylation because of mutation of Ser^{302} to Ala might partially diminish the transduction of growth signals from IRS-1 to the PI3K/mTOR/S6K pathway, which reduces cell proliferation.

In summary, we showed that insulin/IGF-I stimulates Ser^{302} phosphorylation of rat IRS-1 through activation of an mTOR-dependent pathway. Ser^{302} phosphorylation of IRS-1 appears to play a permissive role in insulin-stimulated IRS-1 tyrosine phosphorylation and signal transduction. This result is important because a defect in a positive regulatory IRS-1 Ser/Thr kinase may be another mechanism contributing to insulin resistance. Future studies will reveal the mechanism(s) by which Ser^{302} phosphorylation promotes IRS-1 function and at identifying additional phospho Ser/Thr residues that regulate IRS-1. The effects of Ser/Thr phosphorylation on IRS-1 signaling are likely to be of critical importance to our full understanding of insulin action and insulin resistance.

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