

Serine/Threonine Phosphorylation of IRS-1 Triggers Its Degradation

Possible Regulation by Tyrosine Phosphorylation

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Insulin receptor substrate (IRS)-1 protein expression is markedly reduced in many insulin-resistant states, although the mechanism for this downregulation is unclear. In this study, we have investigated the early events in the insulin pathway that trigger the degradation of IRS-1. Incubation of the adipocytes with insulin induced a fast electrophoretic mobility shift of IRS-1 and a subsequent degradation of the protein. Wortmannin and rapamycin blocked this mobility shift of IRS-1, maintained the insulin-induced tyrosine phosphorylation of IRS-1, and blocked its degradation. In contrast, a glycogen synthase kinase 3 inhibitor, a mitogen-activated protein kinase/extracellular-regulated kinase inhibitor, and various protein kinase C inhibitors had no effect. Incubation with okadaic acid increased the serine/threonine phosphorylation of IRS-1 and its degradation, mimicking insulin, and its effect was prevented by the proteasome inhibitor lactacystin, as well as by rapamycin. Treatment of the cells with the tyrosine phosphatase inhibitor orthovanadate in the presence of insulin or okadaic acid partially inhibited the degradation of IRS-1. We propose that a rapamycin-dependent pathway participates as a negative regulator of IRS-1, increasing its serine/threonine phosphorylation, which triggers degradation. Thus, regulation of serine/threonine versus tyrosine phosphorylation may modulate IRS-1 degradation, affecting insulin sensitivity. *Diabetes* 50:24–31, 2001

Insulin action is initiated through hormone binding to cell-surface insulin receptors, which activates the protein kinase associated with the β -subunit (1). Stimulation of receptor kinase activity induces tyrosine phosphorylation of a variety of endogenous substrates, including insulin receptor substrate (IRS)-1, -2, -3, and -4 (2–5),

which allows them to interact with and recruit Src homology 2 (SH2)-domain-containing proteins, including phosphatidylinositol (PI) 3-kinase, Grb2/mSos, SH-PTP2 (protein tyrosine phosphatase with Src homology 2 domains), Nck, and cFyn (6–9). These events lead to activation of multiple signaling pathways required for insulin's pleiotropic action, resulting in the promotion of glucose uptake, glycogen synthesis, mitogenesis, or gene expression (10).

Type 2 diabetes is characterized by abnormalities of insulin secretion and by insulin resistance in the major target tissues producing a diminished uptake and metabolism of glucose (11). Although the defect in insulin-stimulated glucose disposal has been well described, the underlying molecular mechanisms are not clear. Alterations in the early steps of insulin signaling have been recognized as an important component of many insulin-resistant states (10). Decreased insulin binding, decreased receptor kinase activity, decreased IRS-1 protein, and decreased IRS-1-associated PI 3-kinase activity have all been demonstrated in liver, muscle, and fat from *ob/ob* mice and other models of type 2 diabetes (12–16). IRS-1 proteins are important intracellular molecules that mediate insulin receptor tyrosine kinase signaling, and an IRS-1-related defect may be one of the causes of insulin resistance. Gene disruption of IRS-1 in mice is associated with impaired insulin-stimulated glucose disposal in vivo and glucose transport in vitro, and the residual insulin sensitivity was shown to be dependent on the presence of the alternative docking protein, IRS-2 (17–19). We have demonstrated that IRS-1 is the main docking protein for PI 3-kinase and the associated increase in glucose uptake in normal human adipocytes (20), which has also been demonstrated in other cell types (21–23). Furthermore, we have shown that fat cells from subjects with type 2 diabetes, but not those with type 1 diabetes, have an impaired insulin effect and a marked reduction in the expression of IRS-1 protein, whereas IRS-2 levels remain the same (20). In addition, decreased IRS-1 protein has been observed in various animal models of insulin resistance (13–16) and in vitro models such as tumor necrosis factor- α treatment (24) of 3T3-L1 cells or chronic stimulation with insulin (25–27).

The basic mechanisms for the regulation of IRS-1 protein levels are unclear. An insulin-resistant state due to defects in signal transduction has been obtained after prolonged insulin treatment of 3T3-L1 adipocytes (25–27). This treatment induces a decrease in IRS-1 due to an enhancement of IRS-1 degradation (28,29) mediated by the proteasome pathway (30). In the present study, we have taken advantage of this model, which mimics the human type 2 diabetic fat cell (low

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Received for publication 20 December 1999 and accepted in revised form 2 October 2000.

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DMEM, Dulbecco's modified Eagle's medium; ERK, extracellular signal-regulated kinase; FBS, fetal bovine serum; GSK3, glycogen synthase kinase 3; IRS, insulin receptor substrate; MAPK, mitogen-activated protein kinase; MEK, MAPK/ERK; mTOR, target of rapamycin; PDGF, platelet-derived growth factor; PI, phosphatidylinositol; PKB, protein kinase B; PKC, protein kinase C; SH2, Src homology 2.

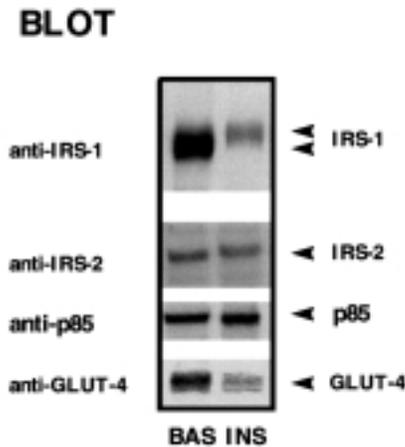


FIG. 1. Effect of chronic insulin treatment on different proteins involved in the insulin-signaling pathway. 3T3-L1 adipocytes were treated in the absence (BAS) or presence (INS) of 100 nmol/l insulin for 18 h. Cells were then lysed, and proteins were separated on 7.5% SDS-PAGE, transferred, and immunoblotted using the indicated antibodies as described in RESEARCH DESIGN AND METHODS. Results are representative of four experiments.

IRS-1 protein levels) (20), to investigate the early events in the insulin pathway that can trigger the degradation of IRS-1 and to find ways to prevent it. We show for the first time that serine/threonine phosphorylation of IRS-1 through a rapamycin-dependent pathway precedes and triggers its degradation by the proteasome and that this process can be prevented by maintaining the tyrosine phosphorylation of the protein.

RESEARCH DESIGN AND METHODS

Cell culture. 3T3-L1 cells were grown and maintained as fibroblasts in Dulbecco's modified Eagle's medium (DMEM) with high glucose containing 10% fetal bovine serum (FBS) in a humidified atmosphere composed of 95% air and 5% CO₂. They were differentiated into adipocytes by being grown in DMEM/high glucose with 10% serum, 0.4 µg/ml dexamethasone, 0.5 mmol/l isobutylmethylxanthine, and 5 µg/ml insulin. After 3 days, the media was changed and the cells were maintained with DMEM containing 10% FBS. After this process, >80% of the cells differentiated into adipocytes.

Chronic insulin treatment. 3T3-L1 adipocytes (7–14 days after confluence) were incubated with DMEM 0.1% FBS with or without insulin (100 nmol/l) (Sigma) or okadaic acid (0.1 µmol/l) (Alexis Corporation, San Diego, CA) at 37°C for the various times. In some cases, lactacystin (50 µmol/l; Calbiochem, La Jolla, CA), wortmannin (1 µmol/l, Sigma), mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) (MEK) inhibitor PD98059 (50 µmol/l), H7 (50 µg/ml), rapamycin (200 nmol/l), the protein kinase C (PKC) zeta inhibitor RO 31-8220 (5 µmol/l) (Calbiochem), LiCl (30 mmol/l), or 2 mmol/l sodium orthovanadate (Sigma) was added for 30 min before the addition of insulin or okadaic acid. At the end of the chronic incubation period, cells were washed with phosphate-buffered saline and lysed. Platelet-derived growth factor (PDGF) (the B/B isoform from Boehringer Mannheim) and endothelin-1 (Sigma) were added as described in the figure legends.

Immunoprecipitations and immunoblotting. After chronic incubation, cells were lysed in 0.4 ml of lysis buffer containing 25 mmol/l Tris-HCl (pH 7.4), 0.5 mmol/l EGTA, 25 mmol/l NaCl, 1% Nonidet P-40, 1 mmol/l Na₃VO₄, 10 mmol/l NaF, 0.2 mmol/l leupeptin, 1 mmol/l benzamide, and 0.1 mmol/l 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride (Calbiochem) and rocked for 40 min at 4°C. Detergent-insoluble material was sedimented by centrifugation at 12,000g for 10 min at 4°C. Cell lysate proteins (50 µg protein) were separated by SDS-PAGE, or 100 µg of protein was immunoprecipitated for 2 h with anti-IRS-1 COOH-terminal antibodies (4 µg/ml; Upstate Biotechnology, Lake Placid, NY). Immune complexes were collected with protein A-Sepharose, washed, solubilized in Laemmli sample buffer, and separated by SDS-PAGE on 7.5% gels. Proteins were transferred from the gel to nitrocellulose sheets and blocked in 5% milk. The blots were probed with various primary antibodies as follows: anti-IRS-1 COOH-terminal, anti-IRS-2, anti-p85 (whole antiserum), anti-ERK, and 4G10 anti-phosphotyrosine antibodies (Upstate Biotechnology), anti-GLUT4 antibodies (Chemicon International, Temecula, CA), anti-phospho

MAPK (Promega), anti-phospho protein kinase B (PKB), and anti-phospho p70 (New England Biolabs, Beverly, MA) according to the recommendations of the manufacturer. The proteins were detected by enhanced chemiluminescence with horseradish peroxidase-labeled secondary antibodies (Amersham). The intensity of the bands was quantitated with a laser densitometer (Molecular Dynamics). Control experiments showed that the antibodies to IRS-1 had a 90% efficiency to deplete the antigen.

Phosphorylation of rat recombinant IRS-1 by cell lysates. Cells were treated with or without rapamycin for 30 min before incubation with 100 nmol/l insulin or 0.1 µmol/l okadaic acid for 3 h and lysed in 100 µl ice-cold 50 mmol/l Tris (pH 7.4) with 0.1 mmol/l 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF) by 15 passages through a 25-gauge needle, as previously described (30). Lysates were centrifuged, and the supernatant fraction was adjusted to Tris 25 mmol/l (pH 7.4), EGTA 0.5 mmol/l, MgCl₂ 10 mmol/l, dithiothreitol 1 mmol/l, 0.1 mg/ml bovine serum albumin, 50 µmol/l cold ATP, 2 µCi [γ-³²P]ATP (ICN, Costa Mesa, CA) containing 1 µg rat recombinant IRS-1 (Upstate Biotechnology, Lake Placid, NY) as a substrate. The in vitro kinase assay was carried out in a final volume of 40 µl for 20 min at 30°C. The reaction was stopped by spotting an aliquot of the samples on phosphocellulose paper filters (Whatman p81, Clifton, NJ), washed in 1% (vol/vol) orthophosphoric acid, and counted in a scintillation counter or by adding 5× Laemmli buffer and boiling for 5 min. Phosphoproteins were resolved by 10% SDS-PAGE, stained, and examined by autoradiography. In some cases, the phosphoproteins corresponding to IRS-1 were excised and counted on a scintillation counter.

RESULTS

Chronic insulin treatment stimulates serine/threonine phosphorylation and degradation of IRS-1 through a wortmannin and rapamycin-sensitive pathway. Chronic hyperinsulinemia induces serine/threonine phosphorylation of IRS-1 and reduces insulin signaling (30,31). Chronic exposure of 3T3-L1 adipocytes to insulin induced a specific reduction of the IRS-1 and GLUT4 proteins (Fig. 1), whereas other proteins, such as IRS-2, PI 3-kinases, and MAPKs (not shown), were unchanged, mimicking the pattern of a type 2 diabetic human fat cell (low IRS-1 and GLUT4 protein) (20).

To further investigate the mechanisms for the downregulation of IRS-1 by insulin and determine the nature of the kinases that catalyze this reaction, 3T3-L1 adipocytes were pretreated for 30 min with various protein kinase inhibitors as indicated, and then insulin was added for various times. Insulin induced a rapid tyrosine phosphorylation of IRS-1 that was reduced after 1.5 h of treatment (Fig. 2B) (30). Figure 2 showed that at 1.5 h, insulin induced a decrease in the electrophoretic mobility shift of IRS-1, which has been demonstrated to be caused by an increase in serine/threonine phosphorylation content (Fig. 2A and B) (32) and a stimulation of PKB and MAPK phosphorylation. Preincubation of the cells with wortmannin blocked the mobility shift of IRS-1 by insulin (Fig. 2A), and this was accompanied by a preservation of the insulin-induced tyrosine phosphorylation of IRS-1 (Fig. 2B). A positive control was performed showing tyrosine phosphorylation of IRS-1 with acute insulin treatment (Fig. 2B, upper panel). In contrast, PD98059, a specific MEK inhibitor, and H7, a general PKC inhibitor, had no effect on the mobility shift of IRS-1 or on the tyrosine phosphorylation of IRS-1. A control Western blot verified that in 3T3-L1 adipocytes insulin was stimulating the formation of phosphorylated MAPK and PKB and that this stimulation was completely inhibited by the MEK inhibitor PD98059 and wortmannin, respectively (Fig. 2A). In addition, only wortmannin inhibited the degradation of the protein after prolonged treatment with insulin (Fig. 2B, lower panel).

Several serine/threonine kinases located downstream of PI 3-kinase are candidates for the serine phosphorylation of IRS-1 and the triggering of the degradation. These include the target of rapamycin (mTOR) (33), p70 S6 kinase (34), PKB (35), atyp-

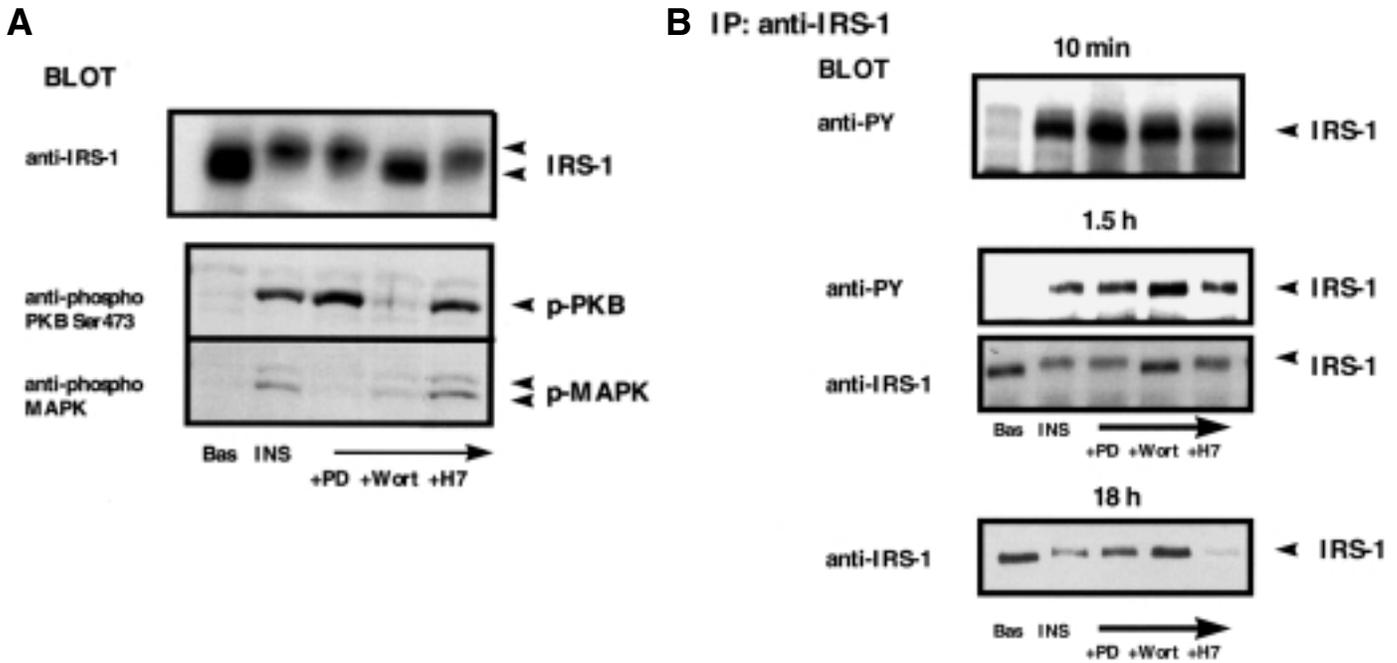


FIG. 2. Effect of inhibitors on insulin-induced phosphorylation and degradation of IRS-1. 3T3-L1 adipocytes were pretreated for 30 min with PD98059 (PD), wortmannin (Wort), or H7 as described in RESEARCH DESIGN AND METHODS. *A*: Next, 100 nmol/l insulin (INS) was added for 1.5 h, cells were lysed, and proteins were separated by 10% SDS-PAGE (long gels) and immunoblotted with the indicated antibodies. *B*: Cells were pretreated with the inhibitors, and then insulin was added for 10 min, 1.5 h, or 18 h. Lysates were immunoprecipitated using anti-IRS-1 antibodies, separated by SDS-PAGE on 7.5% gels, and immunoblotted with the indicated antibodies. The results are representative of four similar experiments.

ical PKCs (36), and glycogen synthase kinase 3 (GSK3) (37). To determine whether any of these kinases could be involved in insulin-induced IRS-1 serine phosphorylation and degradation, 3T3-L1 adipocytes were incubated with different kinase inhibitors before their incubation with insulin. Preincubation of the cells with rapamycin, an inhibitor of mTOR, eliminated both the mobility shift and the degradation of the protein observed after treatment with insulin (Fig. 3A) and preserved the insulin-induced IRS-1 tyrosine phosphorylation (Fig. 3B), without having any effect on insulin receptor tyrosine phosphorylation (not shown). A control blot verified that insulin stimulated the phosphorylation of p70 S6 kinase and that this stimulation was completely inhibited by rapamycin. In contrast, RO 31-8220, an inhibitor of atypical PKCs (36), and lithium chloride, an inhibitor of GSK3, had no such protective effect. These findings suggest that IRS-1 serves as a substrate for an insulin-stimulated kinase whose activity triggers the phosphorylation and degradation of IRS-1 through a wortmannin-rapamycin-sensitive pathway.

Serine/threonine phosphorylation of IRS-1 by okadaic acid triggers its degradation. Treatment of 3T3-L1 adipocytes or U266 cells with the serine threonine phosphatase inhibitor okadaic acid results in increased IRS-1 serine phosphorylation, reduced IRS-1-insulin receptor association, and reduced insulin receptor-dependent tyrosine phosphorylation of IRS-1 (38,39).

To investigate whether okadaic acid can also trigger the degradation of IRS-1, 3T3-L1 adipocytes were incubated for 18 h with this specific serine threonine phosphatase inhibitor. Okadaic acid induced a decrease in the electrophoretic mobility of IRS-1, followed by the degradation of the protein (Fig. 4A). Interestingly, okadaic acid also stimulated the serine/threonine phosphorylation (decrease in the electro-

phoretic mobility) but not the degradation of IRS-2, mimicking the effect of insulin (Fig. 1).

We have found that the insulin-induced degradation of IRS-1 is through a wortmannin-rapamycin-sensitive pathway. To determine whether the same pathway could be involved in the okadaic acid-stimulated degradation of IRS-1, 3T3-L1 cells

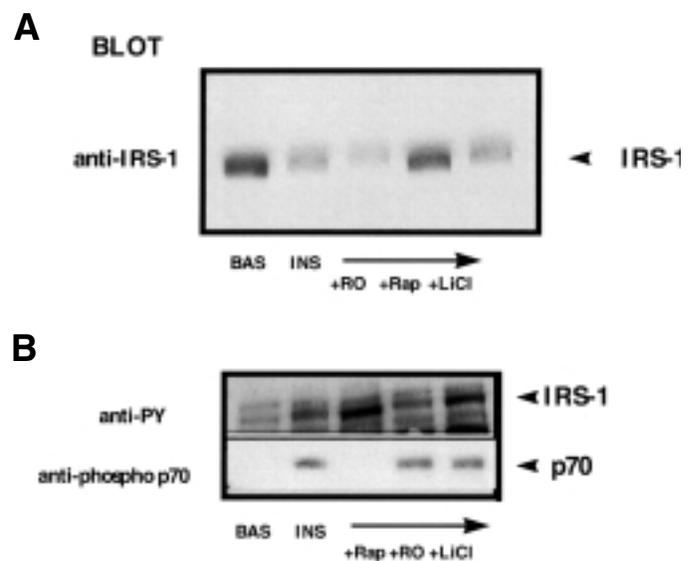


FIG. 3. Effect of inhibitors of kinases downstream of PI 3-kinase on insulin-induced degradation of IRS-1. 3T3-L1 adipocytes were pretreated with LiCl, RO318220 (RO), or rapamycin (Rap) for 30 min before addition of 100 nmol/l insulin (INS). Cells were lysed 18 h (*A*) or 4 h (*B*) after the addition of insulin. Protein lysates were separated in a 7.5% SDS-PAGE, transferred, and immunoblotted with the indicated antibodies.

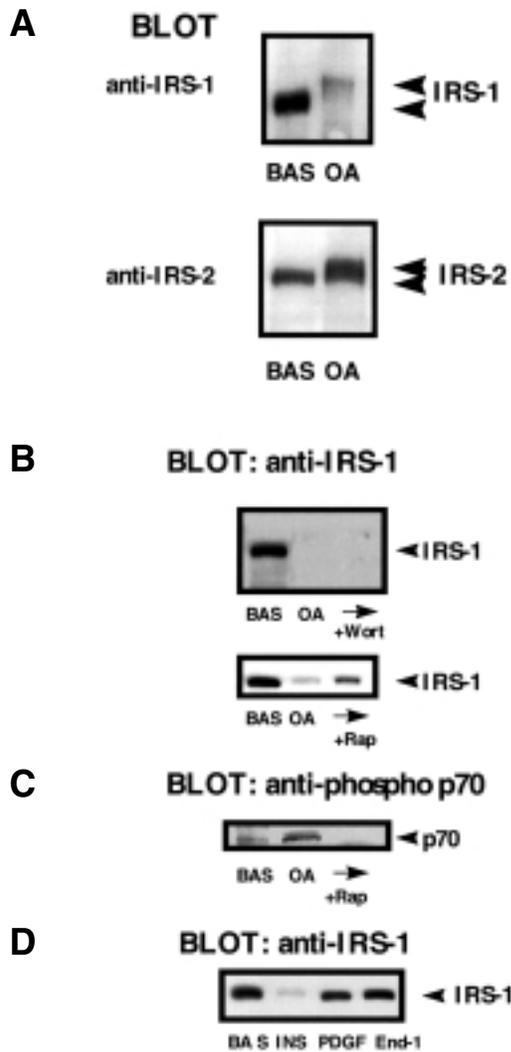


FIG. 4. Serine/threonine phosphorylation of IRS-1 by okadaic acid (OA) triggers the degradation of IRS-1 through a rapamycin-sensitive pathway. **A:** 3T3-L1 cells were treated with or without 0.1 $\mu\text{mol/l}$ okadaic acid for 18 h. **B:** Cells were pretreated with wortmannin (Wort) or rapamycin (Rap) before the addition of 0.1 $\mu\text{mol/l}$ okadaic acid for 18 h. **C:** Cells were pretreated with rapamycin (Rap) before the addition of 0.1 $\mu\text{mol/l}$ okadaic acid for 3 h. **D:** Cells were treated with or without 100 nmol/l insulin (INS), 50 ng/ml PDGF, or 1 $\mu\text{mol/l}$ endothelin-1 (End-1) for 18 h. Cells were lysed, separated on 7.5% SDS-PAGE, and transferred, and IRS-1 or IRS-2 protein levels or phosphorylation of p70 was detected by immunoblotting with specific antibodies.

were pretreated in the absence or presence of wortmannin or rapamycin before incubation with okadaic acid (Fig. 4B). Interestingly, preincubation of the cells with rapamycin, but not with wortmannin, partially inhibited the degradation of IRS-1 by okadaic acid, suggesting that mTOR was involved in its pathway. Okadaic acid also increased the phosphorylation of p70 S6 kinase, and this effect was blocked by rapamycin (Fig. 4C).

Increased serine phosphorylation of IRS-1 has also been observed after treatment of the cells with activators of PKC, PDGF, or endothelin-1 or activation of cellular stress pathways (35,39–41). Because prior studies have indicated that PDGF and endothelin-1 increase the serine phosphorylation of IRS-1 and induce insulin resistance in adipocytes (35), we tested

whether these hormones would also induce degradation of the protein. As shown in Fig. 4D, incubation of the cells for 18 h with PDGF or endothelin-1 had no effect on the degradation of IRS-1, suggesting that specific serine phosphorylation sites could be involved in the degradation of IRS-1. Control experiments showed that these hormones were active (not shown). **Rapamycin inhibits protein kinase activity in chronic insulin- or okadaic acid-treated cells.** To determine whether rapamycin could also inhibit serine/threonine kinase activity responsible for the serine/threonine phosphorylation of IRS-1 in chronic insulin- or okadaic acid-treated cells, lysates were prepared from 3T3-L1 adipocytes pretreated in the presence or absence of rapamycin before incubation with 0.1 $\mu\text{mol/l}$ okadaic acid or 100 nmol/l insulin for 3 h. Lysates were used as sources of kinase to phosphorylate recombinant IRS-1 in an in vitro kinase assay (38). Figure 5 shows that insulin and okadaic acid treatment significantly enhanced kinase activity for recombinant IRS-1, in agreement with the concept that both agents increased the serine/threonine phosphorylation of IRS-1. Moreover, rapamycin inhibited insulin- or okadaic acid-induced serine/threonine phosphorylation of IRS-1, suggesting the presence of a protein kinase downstream of mTOR that phosphorylated IRS-1 in vivo. In contrast, rapamycin did not inhibit insulin-induced tyrosine phosphorylation of IRS-1 (not shown).

Proteasome is involved in IRS-1 degradation by insulin and okadaic acid. Insulin-induced IRS-1 degradation can be prevented by pretreatment with lactacystin, a specific inhibitor for proteasome degradation (30). To investigate whether the mechanism of the okadaic acid-induced IRS-1

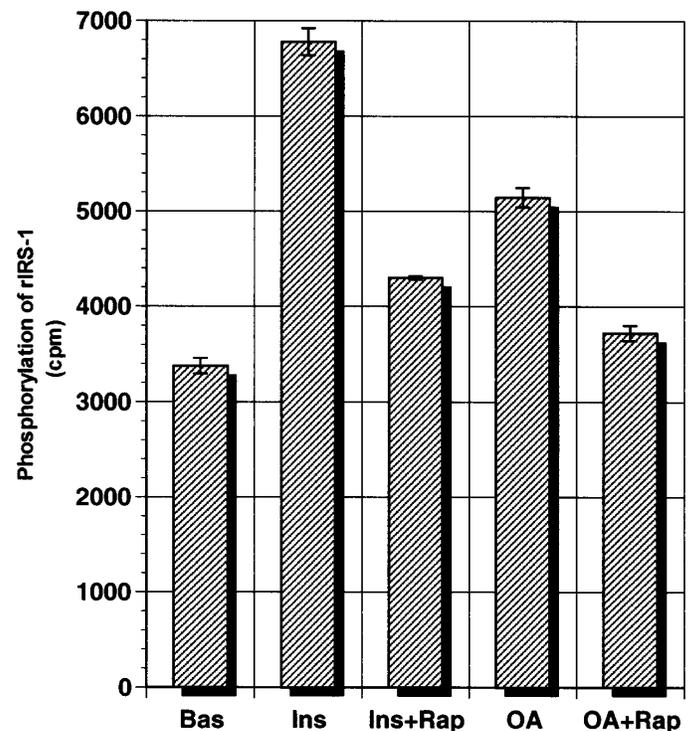


FIG. 5. Phosphorylation of recombinant IRS-1 by cell lysates. Cell lysates were prepared from 3T3-L1 adipocytes pretreated with rapamycin (Rap) before the addition of 100 nmol/l insulin (Ins) or 0.1 $\mu\text{mol/l}$ okadaic acid (OA) for 3 h. In vitro kinase assays using these lysates were performed using recombinant IRS-1 as a substrate as indicated. Data represent means \pm SE of three separate experiments.

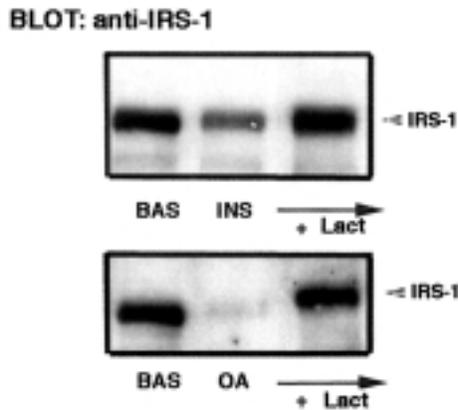


FIG. 6. Proteasome is involved in IRS-1 degradation. Cells were pretreated with 50 $\mu\text{mol/l}$ lactacystin (Lact) for 30 min before treatment with 100 nmo/l insulin (INS) or 0.1 $\mu\text{mol/l}$ okadaic acid (OA). After 18 h, cells were lysed, separated on 7.5% SDS-PAGE, and transferred, and IRS-1 protein levels were detected by immunoblotting with specific antibodies. The blot is representative of three similar experiments.

degradation was also through a proteasome pathway, cells were pretreated in the presence or absence of the specific proteasome inhibitor lactacystin before chronic insulin or okadaic acid treatment. Figure 6 shows that lactacystin completely blocked IRS-1 degradation induced by insulin or okadaic

acid treatment, apparently without changing the mobility shift of the protein. Similar results were found with the specific proteasome inhibitor MG132 (not shown).

Orthovanadate partially prevents the degradation of IRS-1. We have found that agents such as wortmannin and rapamycin, which decrease the degradation of IRS-1, also preserve tyrosine phosphorylation of the protein, possibly by inhibiting tyrosine dephosphorylation of specific sites on IRS-1. To test the hypothesis that tyrosine phosphorylation could prevent the degradation of IRS-1, cells were pretreated with orthovanadate, a potent tyrosine phosphatase inhibitor that is known to increase IRS-1 tyrosine phosphorylation (42; D.L.K., C.M.R., unpublished observations) before treatment with insulin or okadaic acid. As shown in Fig. 7A and B, vanadate partially inhibited the degradation of IRS-1 in IRS-1 immunoprecipitations from cells treated with insulin or okadaic acid for 18 h, and the remaining protein was maintained tyrosine phosphorylated. To investigate whether the same concentration of vanadate (causing IRS-1 tyrosine phosphorylation) was efficient to inhibit IRS-1 degradation by insulin and okadaic acid, cells were cultured for 2 h with different concentrations of vanadate before the addition of insulin or okadaic acid. Insulin induced a decrease in IRS-1 protein levels, as described previously, and this effect was efficiently inhibited by 0.2 mmol/l vanadate, which also produced an increase in IRS-1 tyrosine phosphorylation. In addition, okadaic acid induced a strong alteration in the mobility shift

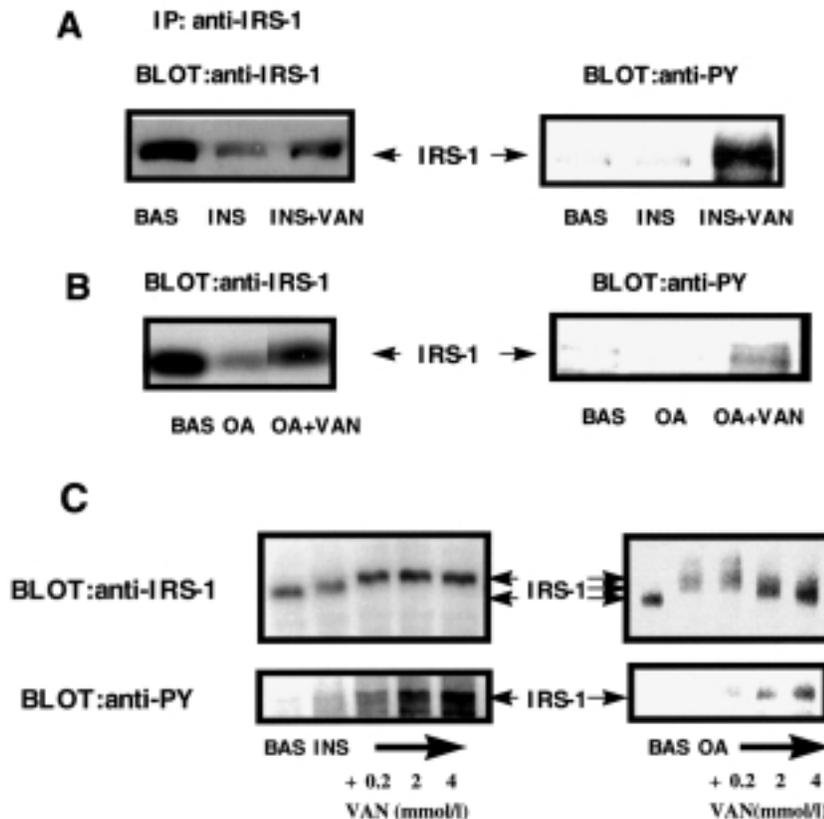


FIG. 7. Effect of orthovanadate on the insulin- or okadaic acid-induced degradation of IRS-1. 3T3-L1 adipocytes were treated with 2 mmol/l sodium orthovanadate (VAN) before the addition of insulin (INS) (A) or okadaic acid (OA) (B) for 18 h. Cells were treated for 2 h with 0.2, 2, or 4 mmol/l sodium orthovanadate (VAN) and 100 nmo/l insulin (INS) or 0.1 $\mu\text{mol/l}$ okadaic acid (OA) (C). Cells were lysed and proteins were immunoprecipitated with antibodies against IRS-1, separated by SDS-PAGE on 7.5% gels, and immunoblotted with the indicated antibodies. Blots are representative of three similar experiments.

of IRS-1 as well as a decrease in protein levels. Vanadate inhibited the mobility shift and the degradation of IRS-1 in a dose-response manner, also increasing tyrosine phosphorylation of the protein. A higher concentration of vanadate was necessary to inhibit okadaic acid-induced IRS-1 degradation, although the tyrosine phosphorylation produced was similar to that induced by a lower concentration of vanadate (0.2 mmol/l) in the presence of insulin.

DISCUSSION

A major negative regulatory role in insulin action is attributed to agents that enhance serine/threonine phosphorylation of IRS-1. Increased serine phosphorylation of IRS-1 has been observed after treatment of cells with activators of PKC, serine/threonine phosphatase inhibitors such as okadaic acid, PDGF, insulin, or angiotensin II or activation of cellular stress pathways by tumor necrosis factor and other cytokines (31,35,39–41,43). Increased serine phosphorylation of IRS-1 has been shown to inhibit the ability of this substrate to be tyrosine phosphorylated by the insulin receptor and to bind and activate PI 3-kinase (31,35). This finding suggests the existence of converging desensitization mechanisms of IRS-1 that involve serine/threonine phosphorylation of IRS-1. A question still unresolved is how this phosphorylation can affect the degradation of IRS-1.

In this study, we present evidence that serine/threonine phosphorylation of IRS-1 precedes degradation of the protein by hyperinsulinemia and that maintaining the tyrosine phosphorylation of IRS-1 may prevent its degradation. Several lines of evidence from the studies presented here support such a hypothesis:

1. Insulin stimulated serine/threonine phosphorylation of IRS-1, reducing its tyrosine phosphorylation and triggering degradation of the protein.
2. Agents such as wortmannin and rapamycin, which blocked the phosphorylation (mobility shift) of IRS-1 and preserved its tyrosine phosphorylation, inhibited IRS-1 degradation.
3. Insulin induced rapamycin-dependent serine/threonine kinase phosphorylated IRS-1 *in vitro*.
4. Okadaic acid, a serine/threonine phosphatase inhibitor, mimicked the effect of insulin, inducing first a serine/threonine phosphorylation of IRS-1 and later its degradation.
5. A general tyrosine phosphatase inhibitor, orthovanadate, preserved tyrosine phosphorylation of IRS-1 and partially inhibited its degradation.

It is known that insulin induces rapid tyrosine phosphorylation of IRS-1, which is followed by a slower increase in its serine/threonine phosphorylation (manifested by reduced mobility) (30,32). Insulin-induced activation of serine/threonine kinases occurs more slowly than the immediate activation of the insulin receptor kinase and the tyrosine phosphorylation of the substrate. This slower activation of kinases, which results in dissociation of IRS-1 from the receptor (31), could serve as a reversible negative-feedback control mechanism to turn off insulin signaling. Although it seems that a redundancy of kinases have the common ability to elevate intracellular serine/threonine phosphate content, only very specific serine/threonine phosphorylation sites seem to be involved in the degradation of IRS-1. The primary sequence of IRS-1 contains at least 35 potential sites for serine/threonine phospho-

rylation by several cytosolic kinases, including protein kinase A, PKC, casein kinase 2, MAPKs, cdc2 kinase, PKB, and GSK3 (2,44). The search for kinases has led to the identification of several candidates based on their ability to phosphorylate IRS-1. Our results showed that rapamycin, an inhibitor of mTOR (a serine/threonine kinase downstream of PI 3-kinase), inhibited the mobility shift of IRS-1, inhibited an elevated serine/threonine kinase activity in chronic insulin- or okadaic acid-treated cells, and later inhibited the degradation of the protein. Moreover, preliminary results showed that rapamycin could restore insulin responsiveness after chronic hyperinsulinemia in 3T3-L1 adipocytes. Interestingly, rapamycin—and not wortmannin—inhibited the degradation of IRS-1 by okadaic acid. This is in agreement with the finding that okadaic acid did not stimulate PI 3-kinase activity (not shown) but did increase the phosphorylation of p70 S6 kinase, and that this effect was inhibited by rapamycin, suggesting that mTOR or a downstream kinase was involved in the degradation of IRS-1 by both insulin and okadaic acid.

We have also found that wortmannin as well as rapamycin preserves the insulin-induced tyrosine phosphorylation of IRS-1. Other studies have shown that treatment of cells with PI 3-kinase inhibitors increases insulin-like growth factor 1-induced tyrosine phosphorylation of IRS-2 with an increase in the mobility of IRS-2, suggesting that PI 3-kinase mediates or is required for IRS-2 serine/threonine phosphorylation, and that this phosphorylation inhibits IRS-2 tyrosine phosphorylation (45). Furthermore, it has been shown previously that wortmannin treatment results in preservation of IRS-1 tyrosine phosphorylation (30,46).

One interesting observation was that PDGF and endothelin-1 did not have any effect on IRS-1 degradation. PDGF has recently been shown to stimulate phosphorylation of serines 632, 662, and 731 in IRS-1 and to inhibit the ability of insulin to stimulate the subsequent tyrosine phosphorylation of IRS-1 and activation of PI 3-kinase (35). Although PDGF can activate mTOR, there is a possibility that the downstream kinase responsible for phosphorylating the specific sites that trigger the degradation is not activated by PDGF or is located in a different compartment. In the same manner, PDGF also activates PI 3-kinase activity but is not efficient to induce glucose transport in adipocytes. In addition, endothelin-1 was found to inhibit the insulin-signaling pathway via the MAPK pathway through the serine phosphorylation of serine 612 in IRS-1. Therefore, our results suggest that serine sites involved in downregulation of insulin signaling are not the same as those that trigger the degradation of IRS-1. These results are again in agreement with the hypothesis that MAPKs are not involved in the degradation of IRS-1 but that potential MAPK phosphorylation sites are likely to be important negative regulators of PI 3-kinase activity associated with IRS-1 (40,41). In addition, we found that okadaic acid induced the degradation of IRS-1, and recent reports have shown that serine/threonine kinases, which are activated after okadaic acid treatment and are responsible for the increase in IRS-1 serine/threonine phosphorylation, are distinct from the MAPKs (40). Moreover, a serine/threonine kinase other than MAPK is activated in the chronic insulin-treated cells and insulin-resistant animals (47).

We have also shown that insulin and okadaic acid increase serine/threonine phosphorylation of IRS-1 and IRS-2 but that only IRS-1 was degraded through a proteasome pathway by these agents, suggesting the presence of specific serine sites

in IRS-1 responsible for its degradation. Although IRS-2 shares substantial structural homology, as well as biological activity, with IRS-1, there are significant structural differences at the COOH-terminal halves of the two molecules (3); therefore, a serine phosphorylation present in the COOH-terminal region of IRS-1, but absent in IRS-2, may be necessary for its degradation. In addition, a novel elevated serine kinase activity that phosphorylates the 721–859 region of IRS-1 was found in lysates from insulin-resistant animals and cell models (47). Another possibility can be the differential compartmentalization and trafficking of IRS-1 and IRS-2 that makes IRS-1 more susceptible to degradation (48).

Vanadate has been demonstrated to improve glucose homeostasis in animal models of type 2 diabetes, and it has been proposed as a potential therapeutic agent for diabetes (49). In vitro, vanadate mimics many insulin actions, although it has been found that vanadium action may not involve the insulin receptor itself but an enhanced tyrosine phosphorylation of IRS-1 (42). In this study, we found that pretreatment with vanadate stimulated tyrosine phosphorylation of IRS-1 and prevented its degradation after insulin or okadaic acid treatment. Chronic stimulation with vanadate also promoted the accumulation of intracellular insulin receptors and inhibited the insulin-induced receptor degradation, possibly because tyrosine dephosphorylation may play a role in the regulation of receptor ligand degradation (50).

IRS-1 serine/threonine phosphorylation may mediate the desensitization of insulin signaling by stimulating the subcellular redistribution of IRS-1 and sensitizing IRS-1 to the action of the proteasome. Serine phosphorylation has been found to be necessary for degradation of many other proteins involved in numerous signaling pathways (51,52). It is important to identify the particular phosphorylated serine sites of IRS-1 that trigger the degradation of the protein as well as the kinases responsible for these phosphorylations.

In summary, we can conclude that IRS-1 serine/threonine phosphorylation, through a rapamycin-sensitive pathway, plays an important role in degradation of the protein and that regulation of tyrosine phosphorylation versus serine/threonine phosphorylation may modulate IRS-1 degradation.

ACKNOWLEDGMENTS

We thank Dr. Terry Opgenorth and Dr. Regina Brun for helpful comments.

REFERENCES

1. Kahn CR, White MF, Shoelson SE, Backer JM, Araki E, Cheatham B, Csermely P, Folli F, Goldstein BJ, Huertas P, Rothenberg PL, Saad MJA, Siddle K, Sun X-J, Wilden PA, Yamada K, Kahn SA: The insulin receptor and its substrate: molecular determinants of early events in insulin action. *Recent Prog Horm Res* 48:291–339, 1993
2. Sun XJ, Rothenberg PL, Kahn CR, Backer JM, Araki E, Wilden PA, Cahill DA, Goldstein BJ, White MF: The structure of the insulin receptor substrate IRS-1 defines a unique signal transduction protein. *Nature* 352:73–77, 1991
3. Sun XJ, Wang LM, Zhang Y, Yenush L, Myers MG Jr, Glasheen EM, Lane WS, Pierce JH, White MF: Role of IRS-2 in insulin and cytokine signaling. *Nature* 377:173–177, 1995
4. Lavan BE, Lane WS, Lienhard GE: The 60-kDa phosphotyrosine protein in insulin-treated adipocytes is a new member of the insulin receptor substrate family. *J Biol Chem* 272:11439–11443, 1997
5. Lavan BE, Fantin VR, Chang ET, Lane WS, Keller SR, Lienhard GE: A novel 160-kD phosphotyrosine protein in insulin-treated embryonic kidney cells is a new member of the insulin receptor substrate family. *J Biol Chem* 272:21403–21407, 1997
6. Myers MG Jr, Backer JM, Sun XJ, Shoelson SE, Hu P, Schlessinger J, Yoakim M, Schaffhausen B, White MF: IRS-1 activates phosphatidylinositol 3'-kinase

- by associating with src homology 2 domains of p85. *Proc Natl Acad Sci U S A* 89:10350–10354, 1992
7. Myers MG Jr, Wang LM, Sun X, Zhang Y, Yenush LP, Schlessinger J, Pierce JH, White MF: Role of IRS-1-GRB-2 complexes in insulin signaling. *Mol Cell Biol* 14:3577–3587, 1994
8. Noguchi T, Matozaki T, Horita K, Fujioka Y, Kasuga M: Role of SH-PTP2, a protein-tyrosine phosphatase with Src homology 2 domains, in insulin-stimulated Ras activation. *Mol Cell Biol* 14:6674–6682, 1994
9. Sun XJ, Pons S, Asano T, Myers MG Jr, Glasheen E, White MF: The Fyn tyrosine kinase binds IRS-1 and forms a distinct signaling complex during insulin stimulation. *J Biol Chem* 271:10583–10587, 1996
10. Virkamäki A, Ueki K, Kahn CR: Protein-protein interaction in insulin signaling and the molecular mechanisms of insulin resistance. *J Clin Invest* 103:931–943, 1999
11. DeFronzo RA, Bonadonna RC, Ferrannini E: Pathogenesis of NIDDM: a balanced overview. *Diabetes Care* 15:318–368, 1992
12. Caro JF, Ittoop O, Pories WJ, Meelheim D, Flickinger EG, Thomas F, Jenquin M, Silverman JF, Khazanie PG, Sinha MK: Studies on the mechanism of insulin resistance in the liver from humans with noninsulin-dependent diabetes: insulin action and binding in isolated hepatocytes, insulin receptor structure, and kinase activity. *J Clin Invest* 78:249–258, 1986
13. Saad MJA, Araki E, Miralpeix M, Rothenberg PL, White MF, Kahn CR: Regulation of insulin receptor substrate 1 in liver and muscle of animal models of insulin resistance. *J Clin Invest* 90:1839–1849, 1992
14. Goodyear LJ, Giorgino F, Sherman LA, Carey J, Smith RJ, Dohm GL: Insulin receptor phosphorylation, insulin receptor substrate-1 phosphorylation, and phosphatidylinositol 3-kinase activity are decreased in intact skeletal muscle strips from obese subjects. *J Clin Invest* 95:2195–2204, 1995
15. Anai M, Funaki M, Ogihara T, Terasaki J, Inukai K, Katagiri H, Fukushima Y, Yazaki Y, Kikuchi M, Oka Y, Asano T: Altered expression levels and impaired steps in the pathway to phosphatidylinositol 3-kinase activation via insulin receptor substrates 1 and 2 in Zucker fatty rats. *Diabetes* 47:13–23, 1998
16. Kerouz NJ, Horsch D, Pons S, Kahn CR: Differential regulation of insulin receptor substrates-1 and -2 (IRS-1 and IRS-2) and phosphatidylinositol 3-kinase isoforms in liver and muscle of the obese diabetic (ob/ob) mouse. *J Clin Invest* 100:3164–3172, 1997
17. Araki E, Lipes MA, Patti ME, Bruning JC, Haag BL III, Johnson RS, Kahn CR: Alternative pathway of insulin signaling in mice with targeted disruption of the IRS-1 gene. *Nature* 372:186–190, 1994
18. Tamemoto H, Kadowaki T, Tobe K, Yagi T, Sakura H, Hayakawa T, Terauchi Y, Ueki K, Kaburagi Y, Satoh S, Sekihara H, Yoshioka S, Horikoshi H, Furuta Y, Ikawa Y, Kasuga M, Yazaki Y, Aizawa S: Insulin resistance and growth retardation in mice lacking insulin receptor substrate-1. *Nature* 372:182–186, 1994
19. Patti ME, Sun XJ, Bruning JC, Araki E, Lipes MA, White MF, Kahn CR: 4PS/IRS-2 is the alternative substrate of the insulin receptor in IRS-1 deficient mice. *J Biol Chem* 270:24670–24673, 1995
20. Rondinone CM, Wang LM, Lonnroth P, Wesslau C, Pierce JH, Smith U: Insulin receptor substrate (IRS) 1 is reduced and IRS-2 is the main docking protein for phosphatidylinositol 3-kinase in adipocytes from subjects with non-insulin-dependent diabetes mellitus. *Proc Natl Acad Sci U S A* 94:4171–4175, 1997
21. Quon MJ, Butte AJ, Zarnowski MJ, Sesti G, Cushman SW, Taylor SF: Insulin receptor substrate 1 mediates the stimulatory effect of insulin on GLUT4 translocation in transfected rat adipose cells. *J Biol Chem* 269:27920–27924, 1994
22. Hara K, Yonezawa K, Sakaue H, Ando A, Kotani K, Kitamura T, Kitamura Y, Ueda H, Stephens L, Jackson TR, Hawkins PT, Dhand R, Clark AE, Holman GD, Waterfield MD, Kasuga M: 1-phosphatidylinositol 3-kinase activity is required for insulin-stimulated glucose transport but not for RAS activation in CHO cells. *Proc Natl Acad Sci U S A* 91:7415–7419, 1994
23. Quon MJ, Chen H, Ing BL, Liu ML, Zarnowski MJ, Yonezawa K, Kasuga M, Cushman SW, Taylor SF: Roles of 1-phosphatidylinositol 3-kinase and ras in regulating translocation of GLUT4 in transfected rat adipose cells. *Mol Cell Biol* 15:5403–5411, 1995
24. Stephens JM, Lee J, Pilch PF: Tumor necrosis factor-alpha-induced insulin resistance in 3T3-L1 adipocytes is accompanied by a loss of insulin receptor substrate-1 and GLUT4 expression without a loss of insulin receptor-mediated signal transduction. *J Biol Chem* 272:971–976, 1997
25. Ricort JM, Tanti JF, Van Obberghen E, Le Marchand-Brustel Y: Alterations in insulin signalling pathway induced by prolonged insulin treatment of 3T3-L1 adipocytes. *Diabetologia* 38:1148–1156, 1995
26. Rice KM, Lienhard GE, Garner CW: Regulation of the expression of pp160, a putative insulin receptor signal protein, by insulin, dexamethasone, and 1-methyl-3-isobutylxanthine in 3T3-L1 adipocytes. *J Biol Chem* 267:10163–10167, 1992
27. Thomson MJ, Williams MG, Frost SC: Development of insulin resistance in 3T3-L1 adipocytes. *J Biol Chem* 272:7759–7764, 1997
28. Rice KM, Turnbow MA, Garner CW: Insulin stimulates the degradation of IRS-1 in 3T3-L1 adipocytes. *Biochem Biophys Res Commun* 190:961–967, 1993

29. Smith LK, Bradshaw M, Croall DE, Garner CW: The insulin receptor substrate (IRS-1) is a PEST protein that is susceptible to calpain degradation in vitro. *Biochem Biophys Res Commun* 196:767-772, 1993
30. Sun XJ, Goldberg JL, Qiao L, Mitchell JJ: Insulin-induced insulin receptor substrate-1 degradation is mediated by the proteasome degradation pathway. *Diabetes* 48:1359-1364, 1999
31. Paz K, Hemi R, LeRoith D, Karasik A, Elhanany E, Kanety H, Zick Y: A molecular basis for insulin resistance: elevated serine/threonine phosphorylation of IRS-1 and IRS-2 inhibits their binding to the juxtamembrane region of the insulin receptor and impairs their ability to undergo insulin-induced tyrosine phosphorylation. *J Biol Chem* 272:29911-29918, 1997
32. Sun XJ, Miralpeix M, Myers MG Jr, Glasheen EM, Backer JM, Kahn CR, White MF: The expression and function of IRS-1 in insulin signal transmission. *J Biol Chem* 267:22662-22672, 1992
33. Scott PH, Brunn GJ, Kohn AD, Roth RA, Lawrence JC Jr: Evidence of insulin-stimulated phosphorylation and activation of the mammalian target of rapamycin mediated by a protein kinase B signaling pathway. *Proc Natl Acad Sci U S A* 95:7772-7777, 1998
34. Ming XF, Burgering BM, Wennstrom S, Claesson-Welsh L, Heldin CH, Bos JL, Kozma SC, Thomas J: Activation of p70/p85 S6 kinase by a pathway independent of p21ras. *Nature* 371:426-429, 1994
35. Li J, DeFea K, Roth RA: Modulation of insulin receptor substrate-1 tyrosine phosphorylation by an Akt/phosphatidylinositol 3-kinase pathway. *J Biol Chem* 274:9351-9356, 1999
36. Standaert ML, Bandyopadhyay G, Perez L, Price D, Galloway L, Poklepovic A, Sajan MP, Cenni V, Sirri A, Moscat J, Toker A, Farese RV: Insulin activates protein kinases C-zeta and C-lambda by an autophosphorylation-dependent mechanism and stimulates their translocation to GLUT4 vesicles and other membrane fractions in rat adipocytes. *J Biol Chem* 274:25308-25316, 1999
37. Eldar-Finkelman H, Krebs EG: Phosphorylation of insulin receptor substrate 1 by glycogen synthase kinase 3 impairs insulin action. *Proc Natl Acad Sci U S A* 94:9660-9664, 1997
38. Cengel FA, Freund GG: JAK1-dependent phosphorylation of insulin receptor substrate-1 (IRS-1) is inhibited by IRS-1 serine phosphorylation. *J Biol Chem* 274:27969-27974, 1999
39. Tanti JF, Gremeaux T, van Obberghen E, Le Marchand-Brustel Y: Serine/threonine phosphorylation of insulin receptor substrate 1 modulates insulin receptor signaling. *J Biol Chem* 269:6051-6057, 1994
40. Mothe I, Van Obberghen E: Phosphorylation of insulin receptor substrate-1 on multiple serine residues, 612, 662, and 731 modulates insulin action. *J Biol Chem* 271:11222-11227, 1996
41. De Fea K, Roth RA: Modulation of insulin receptor substrate-1 tyrosine phosphorylation and function by mitogen-activated protein kinase. *J Biol Chem* 272:31400-31406, 1997
42. Pandey SK, Anand-Srivastava MB, Srivastava AK: Vanadyl sulfate-stimulated glycogen synthesis is associated with activation of phosphatidylinositol 3-kinase and is independent of insulin receptor tyrosine phosphorylation. *Biochemistry* 37:7006-7014, 1998
43. Hotamisligil GS, Peraldi P, Budavari A, Ellis R, White MF, Spiegelman BM: IRS-1-mediated inhibition of insulin receptor tyrosine kinase activity in TNF-alpha- and obesity-induced insulin resistance. *Science* 271:665-668, 1996
44. Sun XJ, Crimmins DL, Myers MG Jr, Miralpeix M, White MF: Pleiotropic insulin signals are engaged by multisite phosphorylation of IRS-1. *Mol Cell Biol* 13:7418-7428, 1993
45. Kim B, Leventhal PS, White MF, Feldman EL: Differential regulation of insulin receptor substrate-2 and mitogen-activated protein kinase tyrosine phosphorylation by phosphatidylinositol 3-kinase inhibitors in SH-SY5Y human neuroblastoma cells. *Endocrinology* 139:4881-4889, 1998
46. Paz K, Liu YF, Shorer H, Hemi R, LeRoith D, Quan M, Kanety H, Seger R, Zick Y: Phosphorylation of insulin receptor substrate-1 (IRS-1) by protein kinase B positively regulates IRS-1 function. *J Biol Chem* 274:28816-28822, 1999
47. Qiao L, Goldberg JL, Russell JC, Sun XJ: Identification of enhanced serine kinase activity in insulin resistance. *J Biol Chem* 274:10625-10632, 1999
48. Inoue G, Cheatham B, Emkey R, Kahn CR: Dynamics of insulin signaling in 3T3-L1 adipocytes: differential compartmentalization and trafficking of insulin receptor substrate (IRS)-1 and IRS-2. *J Biol Chem* 273:11548-11555, 1998
49. Goldfine AB, Simonson DC, Folli F, Patti ME, Kahn CR: Metabolic effects of sodium metavanadate in humans with insulin-dependent and noninsulin-dependent diabetes mellitus in vivo and in vitro studies. *J Clin Endocrinol Metab* 80:3311-3320, 1995
50. Fantus IG, George R, Tang S, Chong P, Poznansky MJ: The insulin-mimetic agent vanadate promotes receptor endocytosis and inhibits intracellular ligand-receptor degradation by a mechanism distinct from the lysosomotropic agents. *Diabetes* 45:1084-1093, 1996
51. Zhang YK, Sun X, Muraoka K, Ikeda A, Miyamoto S, Shimizu H, Yoshioka K, Yamamoto KI: Immunosuppressant FK506 activates NF-kappaB through the proteasome-mediated degradation of I-kappaBalpha: requirement for I-kappaBalpha n-terminal phosphorylation but not ubiquitination sites. *J Biol Chem* 274:34657-34662, 1999
52. Saito T, Ishiguro K, Onuki R, Nagai Y, Kishimoto T, Hisanaga S: Okadaic acid-stimulated degradation of p35, an activator of CDK5, by proteasome in cultured neurons. *Biochem Biophys Res Commun* 252:775-778, 1998