THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 276, No. 43, Issue of October 26, pp. 40362–40367, 2001
© 2001 by The American Society for Biochemistry and Molecular Biology, Inc.
Printed in U.S.A.

Regulation of Insulin/Insulin-like Growth Factor-1 Signaling by Proteasome-mediated Degradation of Insulin Receptor Substrate-2*

Received for publication, June 11, 2001, and in revised form, July 27, 2001
Published, JBC Papers in Press, August 23, 2001, DOI 10.1074/jbc.M105332200

Liangyou Rui‡, Tracey L. Fisher, Jeffrey Thomas, and Morris F. White§

From the Howard Hughes Medical Institute, Joslin Diabetes Center, Harvard Medical School, Boston, Massachusetts 02215

Insulin and insulin-like growth factor-1 (IGF-1) regulate metabolism and body growth through homologous receptor tyrosine kinases that phosphorylate the insulin receptor substrate (IRS) proteins. IRS-2 is an important IRS protein, as it mediates peripheral insulin action and β-cell survival. In this study, we show that insulin, IGF-1, or osmotic stress promoted ubiquitin/proteasome-mediated degradation of IRS-2 in 3T3-L1 cells, Fao hepatoma, cells and mouse embryo fibroblasts; however, insulin/IGF-1 did not promote degradation of IRS-1 in 3T3-L1 preadipocytes or mouse embryo fibroblasts. MG132 or lactacystin, specific inhibitors of 26S proteasome, blocked insulin/IGF-1-induced degradation of IRS-2 and enhanced the detection of ubiquitinated IRS-2. Insulin/IGF1-induced ubiquitination and degradation of IRS-2 was blocked by inhibitors of phosphatidylinositol 3-kinase (wortmannin or LY294002) or mTOR (rapamycin). Chronic insulin or IGF-1 treatment of IRS-1-deficient mouse embryo fibroblasts inhibited IRS-2-mediated activation of Akt and ERK1/2, which was reversed by lactacystin pretreatment. By contrast, IRS-1 activation of Akt and ERK1/2 was not inhibited by chronic insulin/IGF-1 stimulation in IRS-2-deficient mouse embryo fibroblasts. Thus, we identified a novel negative feedback mechanism by which the ubiquitin/proteasome-mediated degradation of IRS-2 limits the magnitude and duration of the response to insulin or IGF-1.

Insulin and insulin-like growth factor 1 (IGF-1)[1] regulate a variety of biological functions through homologous tyrosine kinases that phosphorylate the insulin receptor substrate (IRS) proteins. IRS proteins mediate signal specificity and diversity in various cellular backgrounds (1). Upon ligand binding, the activated insulin/IGF-1 receptors engage IRS proteins and promote the phosphorylation of multiple tyrosine residues, which activate various downstream signaling pathways, including the phosphatidylinositol (PI) 3-kinase and MAP kinase cascades (1, 2). These signaling pathways coordinate a network of protein kinases that regulate the activity of cytoplasmic enzymes and nuclear transcription factors (1–4). Four IRS proteins are known, including IRS-1 and IRS-2, which play a central role in many tissue and organ systems. Deletion of IRS-1 in mice impairs somatic growth and causes peripheral insulin resistance, but diabetes rarely develops owing to lifelong compensatory hyperinsulinemia (5, 6). By contrast, IRS-2 is essential for normal nutrient homeostasis because it mediates both peripheral insulin action and the effect of IGF-1 on β-cell growth; mice lacking IRS-2 fail to maintain sufficient compensatory insulin secretion and develop diabetes as young adults (7). Moreover, female mice lacking IRS-2 are hyperplasic and infertile, owing to a failure of the hypothalamic-pituitary-ovarian axis (8).

Many mechanisms are proposed to explain the inhibition of IRS protein signaling, including phosphotyrosine dephosphorylation, serine/threonine phosphorylation, and degradation (9–14). Recent studies suggest that proteasome-mediated degradation of IRS-1 might be involved in the down-regulation of signaling by insulin and IGF-1 and contribute to insulin resistance (12, 13, 15–17). However, there is no convincing evidence showing that the decrease of endogenous IRS-1 via the ubiquitin/proteasome system contributes to an inhibition of insulin signaling.

Proteasome-mediated degradation regulates many biological processes including gene transcription and cell cycle progression (18–20). Proteins targeted for destruction by 26 S proteasome are usually ubiquitinated by a complex containing an ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2), and ubiquitin-protein ligase (E3) (20–22). In certain cases, phosphorylation promotes ubiquitination and degradation of signaling proteins, including IκBα and β-catenin (18, 23–25). Overexpressed recombinant IRS-1 is ubiquitinated in response to IGF-1, and PI 3-kinase inhibitors block IRS-1 degradation (12, 13, 15–17). However, it is unclear whether insulin promotes ubiquitination of endogenous IRS proteins or whether the ubiquitin/proteasome system inhibits IRS protein signaling. In this work, we show that insulin and IGF-1 stimulate ubiquitination and degradation of IRS-2 in multiple cell types via a PI 3-kinase/Akt/mTOR-dependent pathway, which correlates closely with the inhibition of insulin signaling. This negative feedback mechanism might limit the magnitude and duration of IRS-2-mediated signals, and contribute to insulin resistance associated with hyperglycemia and hyperinsulinemia.

* This work was supported in part by National Institutes of Health Grant DK 43808 (to M. F. W.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Supported by an Individual National Research Service Award from the National Institutes of Health.

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

[1] The abbreviations used are: IGF-1, insulin-like growth factor 1; IRS, insulin receptor substrate; PI, phosphatidylinositol; MEF, mouse embryo fibroblast; MAP, mitogen-activated protein; DMEM, Dulbecco's modified Eagle's medium; E1, ubiquitin-activating enzyme; E2, ubiquitin carrier protein; E3, ubiquitin-protein isopeptide ligase; FBS, fetal bovine serum; PBS, phosphate-buffered saline; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; PAGE, polyacrylamide gel electrophoresis; EGF, epidermal growth factor; PDGF, platelet-derived growth factor; ERK, extracellular signal-regulated kinase.

This paper is available on line at http://www.jbc.org

EXPERIMENTAL PROCEDURES

Reagents—Human insulin and IGF-1 were a gift from Eli Li. Protein A-agarose was purchased from Repligen; 3-isobutylmethylxanthine, dexamethasone, aprotinin, and leupeptin were purchased from Sigma. Enhanced chemiluminescence (ECL) detection system was purchased from Amersham Pharmacia Biotech. Lactacystin, MG132, rapamycin, PD98059, LY294002, wortmannin, and Nonidet P-40 were purchased from Calbiochem. Polyclonal anti-active ERK1/2 (αpERK) was purchased from Promega. Polyclonal anti-phospho-Akt (on Ser-473) (αpAkt) was purchased from New England Biolabs Inc. (Beverly, MA); monoclonal anti-ubiquitin was purchased from Santa Cruz Inc; polyclonal anti-IRS-1 antibodies were raised against the full-length rat IRS-1 (JD#159, used at a dilution of 1:15,000 for immunoblotting). Polyclonal anti-IRS-2 antibodies were raised in the laboratory against amino acids 976–1094 (JD#110) or 618–747 (JD#101) of rat IRS-2. Polyclonal anti-p85 antibodies were raised against the N-terminal SH2 domain of p85 regulatory subunit of PI 3-kinase.

Cell Culture and Differentiation—Fao cells were grown at 37 °C in 5% CO_2 in RPMI 1640 supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin, and 10% fetal bovine serum (FBS). 3T3-L1 preadipocytes were grown at 37 °C in 10% CO_2 in Dulbecco's modified Eagle's medium (DMEM) containing 25 mM glucose, 100 units/ml penicillin, 100 μg/ml streptomycin, and 10% calf serum (HyClone Laboratories, Inc). For adipocyte differentiation, confluent preadipocytes were cultured for 3 days in differentiation medium (DMEM supplemented with 25 mM glucose, 1 μM insulin, 0.5 mM 3-isobutylmethylxanthine, 1 μM dexamethasone, and 10% FBS), and 3 days in DMEM supplemented with 1 μM insulin and 10% FBS. The cells were then grown for an additional 4–9 days in DMEM containing 25 mM glucose and 10% FBS without any other additives (>90% cells are adipocytes).

Preparation of Immortalized Mouse Embryo Fibroblasts (MEF)—The generation of IRS-1 and IRS-2 knockout mice has been described previously (5–7). $IRS1^{-/-}$ or $IRS2^{-/-}$ embryos were harvested on day 16 of gestation. After removal of the head and all internal organs, embryo carcasses were washed with PBS, minced with scissors, and incubated with 0.25% trypsin on ice overnight. The embryos were incubated for an additional 20 min at 37 °C to activate trypsin. Growth medium (DMEM supplemented with 25 mM glucose, 10% heat-inactivated FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin) was added to stop trypsin action, and the embryos were disrupted by vigorous pipetting. The resulting single cell suspension was centrifuged for 6 min at 1000 rpm. The cell pellet was resuspended in growth medium and cultured at 37 °C in 5% CO_2. The MEF cells reached confluence in 2 days and were replated every 72 h at a density of 5×10^3 cells/cm^2 until the establishment of permanent cell lines.

Immunoprecipitation and Immunoblotting—Confluent cells were deprived of serum overnight in DMEM (for 3T3-L1 and MEF cells) or RPMI 1640 (for Fao cells) containing 0.5% bovine serum albumin, and treated with different ligands at 37 °C. The cells were rinsed three times with ice-cold PBSV (10 mM sodium phosphate, pH 7.4, 150 mM NaCl, 1 mM Na_3VO_4), solubilized in lysis buffer (50 mM Tris, pH 7.5, 1% Nonidet P-40, 150 mM NaCl, 2 mM EGTA, 1 mM Na_3VO_4, 100 mM NaF, 10 mM $Na_4P_2O_7$, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin), and centrifuged at 14,000 × *g* for 10 min at 4 °C. The supernatant (cell lysates) was boiled for 5 min in SDS-PAGE sample buffer (50 mM Tris-Cl, pH 6.8, 2% SDS, 10% glycerol, 2% β-mercaptoethanol, and 0.004% bromphenol blue) and separated by SDS-PAGE. In some experiments, the supernatant was incubated with the indicated antibody on ice for 2 h. The immune complexes were collected on protein A-agarose during a 1-h incubation at 4 °C. The beads were washed three times with washing buffer (50 mM Tris, pH 7.5, 1% Nonidet P-40, 150 mM NaCl, 2 mM EGTA) and boiled for 5 min in SDS-PAGE sample buffer. The solubilized proteins were separated by SDS-PAGE. Proteins on the gel were transferred to nitrocellulose membrane (Amersham Pharmacia Biotech) and detected by immunoblotting with the indicated antibody using ECL. Some membranes were subsequently incubated at 55 °C for 30 min in stripping buffer (100 mM β-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl, pH 6.7) to prepare them for a second round of immunoblotting. To make clear cell lysates, cells were scraped in PBS, centrifuged at 14,000 × *g* for 1 min at 4 °C, and boiled for 5 min in SDS-PAGE sample buffer.

RESULTS

Insulin, IGF-1, and Osmotic Stress Reduce IRS-2 Protein Levels—To determine whether insulin and IGF-1 decrease IRS-2 protein levels, 3T3-L1 preadipocytes were treated for 6 h with either insulin (100 nM) or IGF-1 (100 ng/ml). Cell lysates prepared in 1% Nonidet P-40 were resolved by SDS-PAGE and analyzed by immunoblotting with polyclonal antibodies against IRS-2 (αIRS2). IRS-2 was detected in control cell lysates, but not in insulin- or IGF-1-treated cells (Fig. 1*A*, *top panel*). To verify that this protein was IRS-2, 3T3-L1 preadipocytes were treated without or with insulin, and proteins in the cell lysates were immunoprecipitated and immunoblotted with αIRS2. Insulin significantly reduced the level of IRS-2 (Fig. 1*B*). By contrast, insulin and IGF-1 only slightly reduced the level of IRS-1, and did not change the level of the p85 regulatory subunit of PI 3-kinase (Fig. 1*A*).

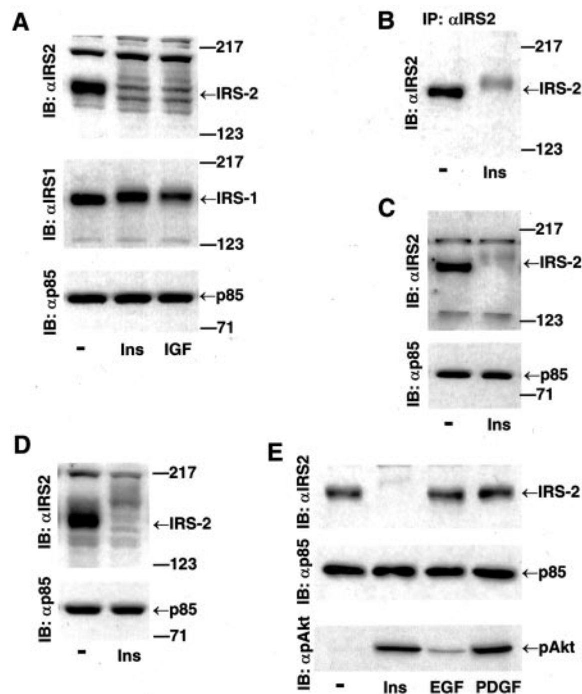

FIG. 1. **Insulin and IGF-1 reduce the level of IRS-2 in 3T3-L1 preadipocytes.** *A*, cells were treated with insulin (*Ins*, 100 nM) or IGF-1 (100 ng/ml) for 6 h and lysed in a buffer containing 1% Nonidet P-40. Cell extracts were resolved by 7% SDS-PAGE and immunoblotted (*IB*) with anti-IRS-2 (#110, against full-length rat IRS-2), αIRS1, or αp85. *B*, cells were treated with insulin and lysed as in *A*. Proteins in cell extracts were immunoprecipitated and immunoblotted with αIRS2. *C*, cells were treated with insulin (100 nM) for 6 h and lysed in a buffer containing 2% SDS. Cell extracts were immunoblotted with αIRS2 or αp85. *D*, cells were treated with insulin (100 nM) for 6 h, and cell extracts were immunoblotted with αp85 or αIRS2 (#101, against the PH domain of rat IRS-2). *E*, cells were treated for 6 h (*top two panels*) or 10 min (*bottom panel*) with insulin (100 nM), EGF (100 ng/ml), or PDGF-BB (50 ng/ml). Cell extracts were immunoblotted with αIRS2, αp85, or antibodies against phosphorylated Akt (αpAkt). The migration of molecular standards, IRS-1, IRS-2, and p85 is indicated.

To exclude the possibility that IRS-2 was redistributed into an inaccessible compartment, insulin-stimulated cells were lysed in buffer containing 2% SDS, and clear lysates were resolved by SDS-PAGE and immunoblotted with αIRS2. Consistent with the initial results, insulin reduced the level of IRS-2 but not p85 (Fig. 1*C*). Similar results were obtained with polyclonal antibodies raised against other regions of IRS-2, excluding the possibility that phosphorylation of IRS-2 inhibited its immunoblotting by αIRS2 (Fig. 1*D*).

The degradation of IRS-2 was tested by other growth factors and in various cell backgrounds. 3T3-L1 preadipocytes were treated with epidermal growth factor (EGF) (100 ng/ml), platelet-derived growth factor-BB (PDGF) (50 ng/ml), or insulin. As expected, insulin stimulated reduction of IRS-2, but neither EGF nor PDGF altered the level of IRS-2; the level of p85 was not changed in response to insulin, EGF, or PDGF (Fig. 1*E*).

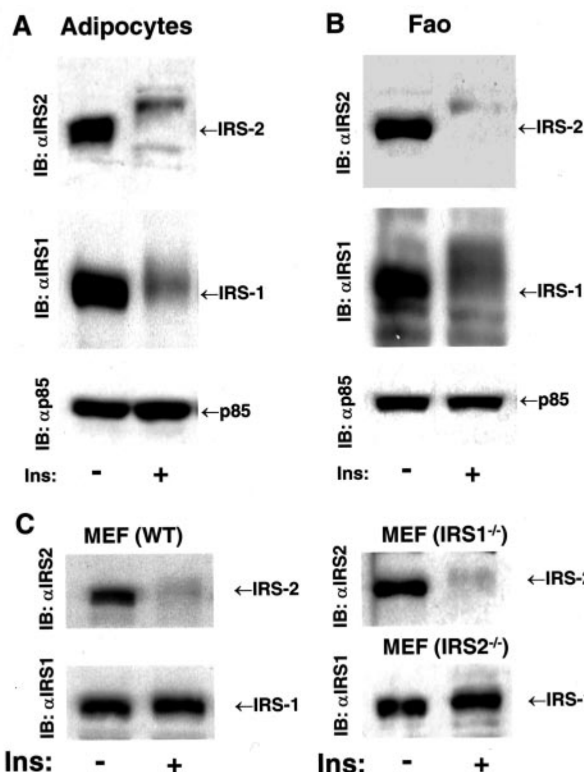

FIG. 2. **Insulin decreases the level of IRS-2 in different cell types.** 3T3-L1 adipocytes (*A*), Fao cells (*B*), and MEF cells derived from wild-type (*WT*), IRS1$^{-/-}$ (*C*) or IRS2$^{-/-}$ mice (*D*) were treated with insulin (*Ins*, 100 nM) for 6 h. Cell extracts were resolved by 7% SDS-PAGE and immunoblotted (*IB*) with αIRS2, αp85, or αIRS1 as indicated.

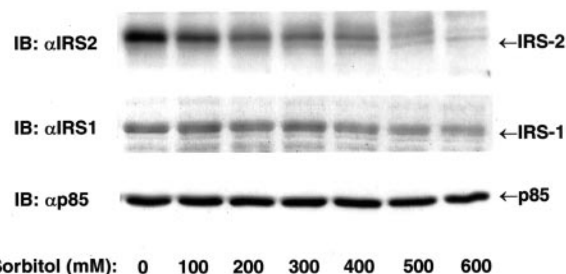

FIG. 3. **Osmotic stress decreases IRS-2 level.** Fao cells were treated for 60 min with the indicated concentration of D-sorbitol. Cell extracts were resolved by 7% SDS-PAGE and immunoblotted (*IB*) with αIRS2, αIRS1, or αp85 as indicated.

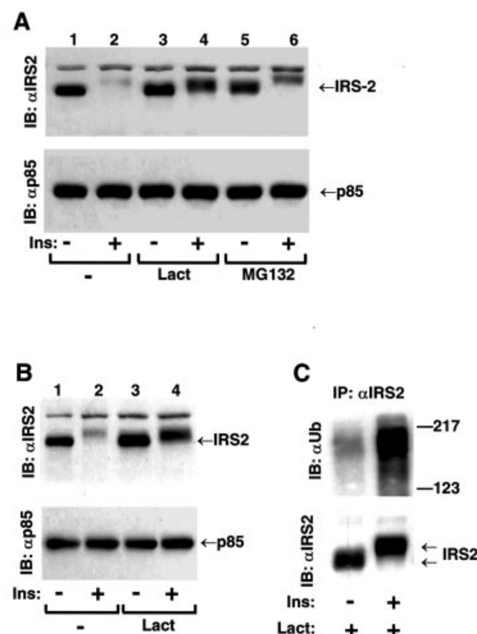

FIG. 4. **Insulin stimulates ubiquitin/proteasome-mediated degradation of IRS-2.** 3T3-L1 preadipocytes (*A*) or Fao cells (*B*) were pre-incubated for 30 min with lactacystin (*Lact*, 10 μM) or MG132 (50 μM) prior to insulin (*Ins*, 100 nM) stimulation for 6 h. Cell extracts were immunoblotted (*IB*) with αIRS2 or αp85. *C*, Fao cells were pretreated with lactacystin (10 μM) for 30 min prior to insulin (100 nM) stimulation for 1 h. IRS-2 was immunoprecipitated with αIRS2 and immunoblotted with αubiquitin. The blot was reprobed with αIRS2.

Both insulin and PDGF strongly stimulated phosphorylation and activation of Akt, whereas EGF stimulated Akt to a less extent (Fig. 1*E*).

Insulin-induced degradation of IRS-1 or IRS-2 was compared in several cell types, including 3T3-L1 adipocytes, Fao hepatoma cells, or MEF cells from wild-type, IRS1$^{-/-}$, or IRS2$^{-/-}$ knockout mice. The cells were treated with insulin (100 nM) for 6 h, and proteins in cell lysates were immunoblotted with αIRS1, αIRS2, or αp85. IRS-2 levels decreased in all three of the cell types, whereas IRS-1 was not reduced in wild-type and IRS2$^{-/-}$ MEF cells; p85 did not change (Fig. 2).

Osmotic stress caused by hyperglycemia might exacerbate peripheral insulin resistance and β-cell dysfunction by impairing insulin/IGF-1 signaling (26). To examine whether osmotic stress induced reduction of IRS-2, Fao cells were treated for 60 min with various concentrations of D-sorbitol. Osmotic stress dramatically reduced the level of IRS-2 but not p85 in a dose-dependent fashion (Fig. 3). It also reduced IRS-1 protein levels, but to a lesser extent (Fig. 3). Together, these results suggest that hyperinsulinemia and/or hyperglycemia might promote degradation of IRS-2 that exacerbates insulin/IGF-1 resistance.

Insulin/IGF-1 Stimulates 26 S Proteasome-mediated Degradation of IRS-2—IRS-2 protein levels decreased after 1 h of insulin treatment and reached the lowest level within 3 h (data not shown). This rapid reduction of IRS-2 was most likely caused by proteolytic degradation rather than by inhibition of transcription and/or translation. To confirm that protein degradation was involved, 3T3-L1 preadipocytes were pretreated without or with lactacystin or MG132, specific inhibitors of 26S proteasome (22). Without drug pretreatment, insulin reduced the levels of IRS-2, as described previously (Fig. 4*A*, *lane 2 versus lane 1*). However, both lactacystin and MG132 inhibited insulin-induced reduction of IRS-2 protein levels (Fig. 4*A*, *upper panel*); lactacystin and MG132 also inhibited IGF-1-induced reduction of IRS-2 (data not shown). Similarly, insulin-induced reduction of IRS-2 was inhibited by lactacystin in Fao cells (Fig. 4*B*) and MEF (data not shown). Lactacystin and MG132 did not alter the level of p85 (Fig. 4, *A* and *B*, *lower panel*). Insulin ordinarily stimulates a shift in mobility of IRS-2, owing largely to phosphorylation (Fig. 4*A*). Lactacystin and MG132 did not significantly affect the insulin-induced mobility shift of IRS-2, suggesting that these inhibitors did not directly inhibit phosphorylation of IRS-2 (Fig. 4, *A* and *B*). Consistent with this idea, lactacystin and MG132 did not alter tyrosyl phosphorylation of IRS-1 and IRS-2 induced during 5 min of insulin stimulation (data not shown). Taken together, the data suggest that 26 S proteasome mediates the degradation of IRS-2 during insulin/IGF-1 stimulation.

Ubiquitination targets proteins for degradation by 26 S proteasome (27). To determine whether insulin promoted the ubiquitination of IRS-2, Fao cells were treated for 1 h with 100 nM insulin. IRS-2 was immunoprecipitated, resolved by SDS-

PAGE, and analyzed by immunoblotting with anti-ubiquitin antibody; however, ubiquitination of IRS-2 was not detected by this experimental protocol. However, when cells were pretreated with lactacystin or MG132, insulin-stimulated ubiquitination of IRS-2 was clearly detected by immunoblotting with anti-ubiquitin, and the migration of ubiquitinated IRS-2 was significantly retarded (Fig. 4*C*). Thus, under ordinary conditions, ubiquitinated IRS-2 might not accumulate to levels sufficient to be detected by immunoblotting, owing to rapid proteasome-mediated degradation.

The PI 3-Kinase → Akt → mTOR Pathway Is Required for Insulin/IGF-1-induced Degradation of IRS-2—IRS proteins mediate the activation of the PI 3-kinase and the MAP kinase cascades during insulin/IGF-1 stimulation (1, 2). To determine whether these signaling pathways mediate degradation of IRS-2, 3T3-L1 preadipocytes or FAO cells were pretreated with wortmannin or LY294002 (inhibitors of type 1b PI 3-kinase), or PD98059 (an inhibitor of MEK1/2). As expected, both wortmannin and LY294002 blocked insulin-stimulated activation of the PI 3-kinase/Akt pathways without altering the activation of the MEK/MAP kinase cascade, whereas PD98059 inhibited insulin-stimulated activation of ERK1/2 without effect on the PI 3-kinase/Akt pathways (data not shown). Moreover, wortmannin and LY294002, but not PD98059, inhibited insulin-induced degradation of IRS-2 (Fig. 5, *A* and *B*). Similarly, wortmannin and LY294002 but not PD98059 inhibited IGF-1-stimulated degradation of IRS-2 in 3T3-L1 preadipocytes (data not shown). These drugs did not change the level of p85 during these experiments (Fig. 5*A*). Consistent with these data, LY294002 inhibited insulin-induced ubiquitination of IRS-2 in the presence of lactacystin (Fig. 5*C*).

mTOR is a serine/threonine kinase downstream of the PI 3-kinase/Akt pathway that contributes to the activation of $p70^{S6}$ kinase (28–32). To determine whether mTOR mediates the degradation of IRS-2, Fao cells were treated prior to insulin stimulation with rapamycin, an inhibitor of mTOR. Rapamycin inhibited insulin-stimulated degradation of IRS-2, as demonstrated by immunoblotting cell extracts with αIRS2 (Fig. 5*B*, *lane 5*). Similarly, rapamycin also blocked IGF-1-stimulated degradation of IRS-2 in 3T3-L1 preadipocytes (data not shown). As expected, rapamycin inhibited the activation of $p70^{S6}$ kinase, as revealed by the inhibition of insulin-induced shift in mobility of $p70^{S6}$ kinase that represents phosphorylation and activation of $p70^{S6}$ kinase (Fig. 5*B*, *lane 5*). Rapamycin and insulin did not alter the level of $p70^{S6}$ kinase (Fig. 5*B*, *lower panel*).

Proteasome-mediated Degradation of IRS-2 Down-regulates Signaling by Insulin and IGF-1—IRS-1 and IRS-2 mediate common and unique signals during insulin/IGF-1 action. Differential degradation of these IRS proteins may have important influence on metabolic regulation. However, this hypothesis is difficult to test in ordinary cell backgrounds that express both IRS-1 and IRS-2. To overcome these technical difficulties, MEF cells derived from IRS-1 and IRS-2 knockout mice were used.

The expression of IRS-1 or IRS-2 was undetectable in $IRS1^{-/-}$ and $IRS2^{-/-}$ MEF cells, respectively (data not shown). Deletion of either IRS-1 or IRS-2 did not alter the level of insulin/IGF-1 receptors (data not shown). Both insulin and IGF-1 promoted activation of Akt as well as ERK1/2 in $IRS1^{-/-}$ or $IRS2^{-/-}$ MEF, as demonstrated by immunoblotting cell extracts with antibodies against phospho-Akt or phospho-ERK, respectively (Fig. 6, *lanes 1–3*). These results suggest that IRS-1 and IRS-2 are redundant in mediating insulin-stimulated activation of the PI 3-kinase/Akt and the MEK → ERK pathways in this cell background. However, insulin stimulated degradation of IRS-2 in $IRS1^{-/-}$ MEF, but insulin did not promote IRS-1 degradation in $IRS2^{-/-}$ MEF cells (Fig. 2*C*).

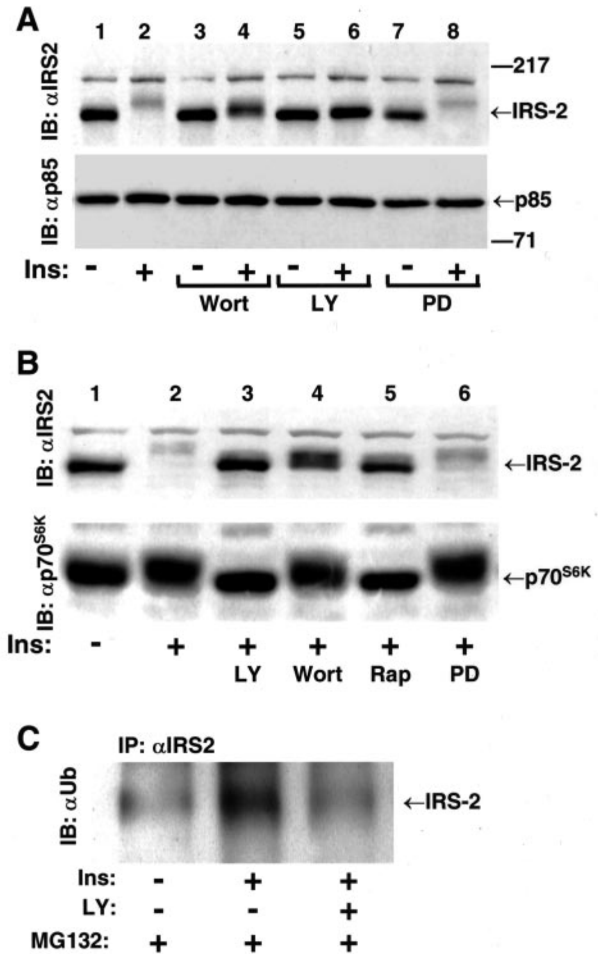

FIG. 5. **The PI 3-kinase/mTOR pathway but not the MEK/EKR1/2 cascade is required for insulin-induced degradation of IRS-2.** *A* and *B*, 3T3-L1 preadipocytes or Fao cells were pretreated for 30 min with 30 μM LY294002 (*LY*), 100 nM wortmannin (*Wort*), 100 μM PD98059 (*PD*), or 10 μM rapamycin (*Rap*), prior to insulin (*Ins*, 100 nM) stimulation for 6 h. Cell extracts were immunoblotted (*IB*) with αIRS2, αp85, or $αp70^{S6K}$. *C*, Fao cells were pretreated with MG132 (50 μM) for 30 min in the absence or presence of LY294002 prior to insulin (100 nM) stimulation for 1 h. Immunopurified IRS-2 was immunoblotted with anti-ubiquitin (*αUb*).

To determine whether degradation of IRS-2 inhibits signaling by insulin and IGF-1, $IRS1^{-/-}$ MEF cells were pretreated for 4 h with 100 nM insulin or 100 ng/ml IGF-1. Cells were then washed, incubated in serum-free medium for additional 2 h, and stimulated for 5 min with 100 nM insulin or 100 ng/ml IGF-1. Activation of the PI 3-kinase → Akt and the MEK → ERK1/2 pathways was measured by immunoblotting cell extracts with antibodies against phospho-Akt or phospho-ERK1/2, respectively. Insulin or IGF-1 pretreatment did not alter the levels of Akt or ERK1/2 in these cells (Fig. 6, *A* and *B*). However, insulin pretreatment significantly reduced the activation of both Akt and ERK1/2 in response to a subsequent insulin or IGF-1 stimulation (Fig. 6, *A* and *B*). Similarly, IGF-1 pretreatment inhibited the activation of Akt and ERK1/2 induced by a subsequent insulin/IGF-1 stimulation to a greater extent (Fig. 6, *A* and *B*, *lanes 7–9*). IGF-1 pretreatment almost completely inhibited activation of Akt induced by subsequent insulin stimulation (Fig. 6*A*, *lane 8*). The difference in the magnitude of inhibition between insulin and IGF-1 might reflect a higher abundance of IGF-1 receptors in MEF cells (data not shown).

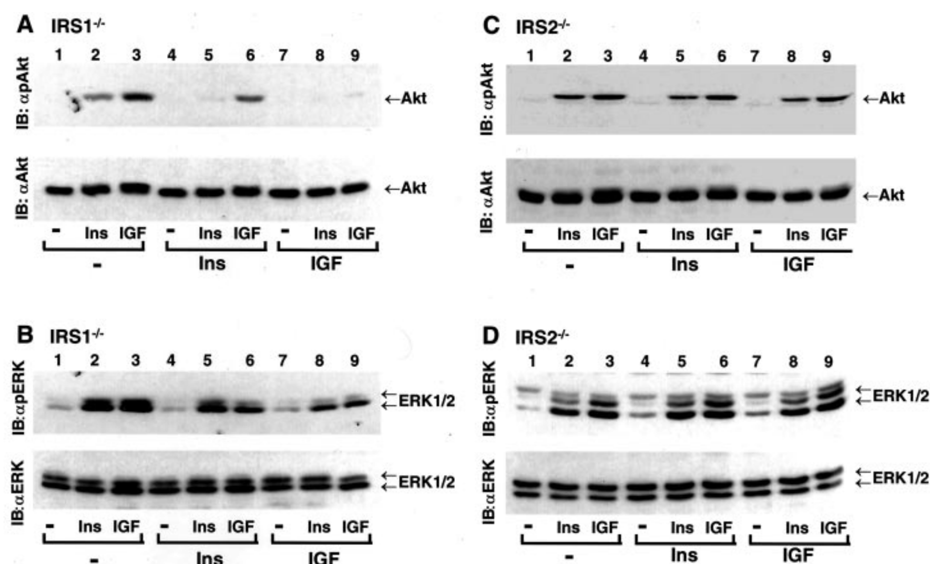

FIG. 6. **Degradation of IRS-2 down-regulates signaling by insulin and IGF-1.** IRS1$^{-/-}$ (*A* and *B*) or IRS2$^{-/-}$ (*C* and *D*) MEF cells were pretreated with insulin (*Ins*, 100 nM) or IGF-1 (*IGF*, 100 ng/ml) for 4 h. The cells were washed with PBS and incubated in serum-free medium for additional 2 h. The treated cells were then stimulated for 5 min with insulin (100 nM) or IGF-1 (100 ng/ml). Cell extracts were resolved by 8% SDS-PAGE and immunoblotted (*IB*) with antibodies against phosphorylated Akt (*αpAkt*) (*A* and *C*) or phosphorylated ERK1/2 (*αpERK*) (*B* and *D*). The same blots were reprobed with αAkt or αERK2 as indicated. αERK2 reacts with both ERK1 and ERK2.

In the parallel experiments with IRS2$^{-/-}$ MEF cells, neither insulin nor IGF-1 pretreatment inhibited the activation of Akt and ERK1/2 induced by a subsequent acute stimulation with insulin or IGF-1 (Fig. 6, *C* and *D*). This resistance to the inhibition by insulin/IGF-1 pretreatment is consistent with the resistance of IRS-1 to degradation in IRS2$^{-/-}$ MEF cells. Moreover, these results revealed that insulin or IGF-1 pretreatment did not inhibit ligand-stimulated activation of the receptors for insulin or IGF-1, nor did it impair the PI 3-kinase/Akt and MEK/ERK1/2 pathways. Therefore, the inhibition of Akt and ERK1/2 by insulin or IGF-1 pretreatment in IRS1$^{-/-}$ MEF cells is most likely caused by insulin/IGF1-induced degradation of IRS-2.

To confirm the hypothesis that proteasome-mediated degradation of IRS-2 contributes to down-regulation of signaling by insulin and IGF-1, IRS1$^{-/-}$ MEF cells were incubated for 30 min with or without the proteasome inhibitor lactacystin prior to IGF-1 pretreatment. IGF-1 pretreatment inhibited activation of Akt and ERK1/2 in response to a subsequent IGF-1 stimulation as expected. However, lactacystin completely reversed the inhibition of Akt and ERK1/2 by IGF-1 pretreatment (Fig. 7).

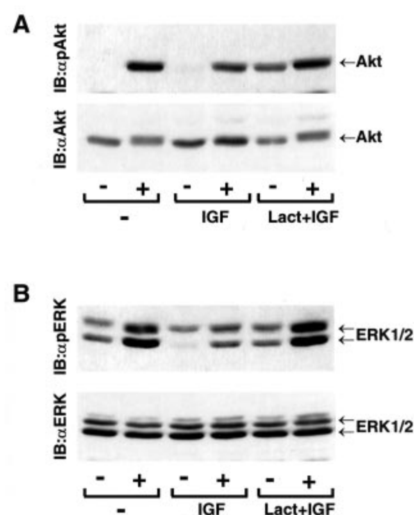

FIG. 7. **Inhibition of proteasome-mediated degradation of IRS-2 improves signaling by IGF-1.** IRS1$^{-/-}$ MEF cells were pretreated for 30 min with or without lactacystin (*Lact*, 30 μM) prior to IGF-1 (*IGF*, 100 ng/ml) stimulation for 4 h. Cells were then washed with PBS and incubated in serum-free medium for additional 2 h. The treated cells were then stimulated with IGF-1 (100 ng/ml) for 5 min. Cell extracts were resolved by 8% SDS-PAGE and immunoblotted (*IB*) with antibodies against phosphorylated Akt (*A*) or ERK1/2 (*B*). The blots were reprobed with anti-Akt or anti-ERK2 as indicated.

DISCUSSION

The causes of prevalent forms of type 2 diabetes are poorly understood. Initially, patients develop mild insulin resistance and glucose intolerance. Hyperglycemia then prompts β-cells to secret compensatory insulin to overcome insulin resistance, resulting in hyperinsulinemia. Although moderate hyperinsulinemia might be well tolerated in the short term, chronic hyperglycemia and hyperinsulinemia exacerbate insulin resistance; if uncontrolled, this process continues until β-cells fail to compensate, resulting in diabetes (3, 4, 26). IRS-2 is critical in both insulin action and β-cell function, as demonstrated by the finding that deletion of IRS-2 in mice causes severe insulin resistance and β-cell failure (7). In ob/ob mice, IRS-2 protein is undetectable in liver, which correlates with hyperglycemia and hyperinsulinemia, hallmarks of insulin resistance (33, 34). Exogenous leptin treatment not only reverses insulin resistance, but also increases concomitantly IRS-2 protein to a normal level (34). Thus, the decrease of hepatic IRS-2 might be a major determinant for overall insulin resistance (35).

In this study, we showed that insulin substantially reduces IRS-2 protein levels in multiple cell lines, which is blocked by specific inhibitors of the 26 S proteasome. These results suggest that proteasome-mediated degradation of IRS-2, rather than inhibition of transcription and/or translation of IRS-2, determines IRS-2 protein levels and activation of IRS-2-mediated signaling pathways. Consistent with this idea, insulin stimulates ubiquitination of IRS-2. During our experiments, the ubiquitinated IRS-2 is detected only in the presence of proteasome inhibitors, suggesting that ubiquitinated IRS-2 is degraded rapidly by the 26 S proteasome. Consistent with these findings, deletion of insulin receptor in liver increases specifically hepatic IRS-2 by more than 5-fold (35), suggesting that the ablation of the insulin signals might block IRS-2 degradation in a whole animal. Reduction of IRS-2 by ubiquitin/proteasome-mediated proteolysis in mouse embryo fibroblasts lacking IRS-1 dramatically inhibits the activation of Akt and ERK1/2 in response to insulin/IGF-1. Strikingly, proteasome inhibitors completely reverse this inhibition. Interestingly, inhibition of the PI 3-kinase → Akt → mTOR pathway, but not the MEK → ERK1/2 pathway, prevents insulin-induced degradation of IRS-2. These observations suggest that the ini-

tial activation of the PI 3-kinase → Akt → mTOR pathway by insulin induces ubiquitination and subsequent degradation of IRS-2 by 26 S proteasome, resulting in inhibition of signaling pathways downstream of IRS-2 during subsequent insulin stimulation. This negative feedback mechanism is likely to modulate insulin action in the whole body by limiting the magnitude and duration of insulin action. Both insulin and osmotic stress stimulate proteasome-mediated degradation of IRS-2, and insulin and osmotic stress exhibit synergistic effect on destruction of IRS-2 (data not shown). Interestingly, the activity of the ubiquitin/proteasome system is elevated in diabetes, suggesting that IRS-2 degradation might be exacerbated (36, 37). Thus, proteasome-mediated degradation of IRS-2 might be involved in the development of insulin resistance and diabetes.

Serine/threonine phosphorylation plays an important role in ubiquitin/proteasome-mediated destruction of signaling proteins. For instance, tumor necrosis factor α stimulates phosphorylation of IκBα at Ser-32 and Ser-36 by IKK complex, which enable β-TrCP, a F-box-containing subunit of the SCF complex, to recognize and bind to phosphorylated IκBα together with E2, resulting in the ubiquitination of IκBα. The ubiquitinated IκBα is subsequently degraded by the 26 S proteasome (19, 23, 24). We propose a similar mechanism for IRS-2 degradation. Insulin or IGF-1 activates the PI 3-kinase → Akt → mTOR pathway. Kinase(s) within this pathway phosphorylate IRS-2, most likely on serines/threonine residues. This phosphorylated motif(s) in IRS-2 might serve as a signal tag for a specific E2·E3 complex with F-box-containing subunits. Consistent with this hypothesis, we and others observed that insulin, IGF-1, and osmotic stress promote serine/threonine phosphorylation of both IRS-1 and IRS-2 (14, 38–41). Serine/threonine phosphorylation of IRS-1 was reported recently to promote its degradation in some cell types (41), and we confirmed insulin-stimulated degradation of IRS-1 in 3T3-L1 adipocytes and Fao cells. However, insulin stimulates degradation of IRS-2 in 3T3-L1 preadipocytes and MEF, but fails to promote IRS-1 degradation. Thus, degradation of IRS-1 and -2 may be mediated by different mechanisms. In addition, these observations raise an intriguing possibility that, in certain insulin/IGF-1 target cells, insulin/IGF-1-promoted degradation of IRS-2 might enhance IRS-1-mediated signaling by reducing the competition by IRS-2 of common signaling molecules including receptors for insulin/IGF-1 and downstream effector molecules; therefore, proteasome-mediated degradation of IRS-2 might positively propagate insulin/IGF-1 signals in some physiological contexts.

In summary, insulin and IGF-1 stimulate substantial degradation of IRS-2 mediated by the ubiquitin/proteasome system in multiple cell types, leading to an inhibition of insulin/IGF-1 signaling. The PI 3-kinase → Akt → mTOR pathway is required for insulin/IGF-1-induced degradation of IRS-2. We propose that the ubiquitin/proteasome-mediated degradation of IRS-2 is a component of negative feedback inhibition modulating the action of insulin and IGF-1. Inhibitors of 26 S proteasome significantly improve insulin sensitivity, suggesting that these types of agents might have clinical value in the treatment of patients with type 2 diabetes.

Acknowledgments—We thank Dr. Xueyin Lin for helpful discussion, and Lauren Kelly and Jack Jackson for assistance in manuscript preparation.

REFERENCES

1. Yenush, L., and White, M. F. (1997) *BioEssays* **19,** 491–500
2. Myers, M. G., Jr., and White, M. F. (1996) *Annu. Rev. Pharmacol. Toxicol.* **36,** 615–658
3. Pessin, J. E., and Saltiel, A. R. (2000) *J. Clin. Invest.* **106,** 165–169
4. Shulman, G. I. (2000) *J. Clin. Invest.* **106,** 171–176
5. 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., and Aizawa, S. (1994) *Nature* **372,** 182–186
6. Araki, E., Lipes, M. A., Patti, M. E., Bruning, J. C., Haag, B., III, Johnson, R. S., and Kahn, C. R. (1994) *Nature* **372,** 186–190
7. Withers, D. J., Gutierrez, J. S., Towery, H., Burks, D. J., Ren, J. M., Previs, S., Zhang, Y., Bernal, D., Pons, S., Shulman, G. I., Bonner-Weir, S., and White, M. F. (1998) *Nature* **391,** 900–904
8. Burks, D. J., de Mora, J. F., Schubert, M., Withers, D. J., Myers, M. G., Towery, H. H., Altamuro, S. L., Flint, C. L., and White, M. F. (2000) *Nature* **407,** 377–382
9. Elchebly, M., Payette, P., Michaliszyn, E., Cromlish, W., Collins, S., Loy, A. L., Normandin, D., Cheng, A., Himms-Hagen, J., Chan, C. C., Ramachandran, C., Gresser, M. J., Tremblay, M. L., and Kennedy, B. P. (1999) *Science* **283,** 1544–1548
10. Goldstein, B. J., Ahmad, F., Ding, W., Li, P. M., and Zhang, W. R. (1998) *Mol. Cell. Biochem.* **182,** 91–99
11. Hotamisligil, G. S. (1999) *Exp. Clin. Endocrinol. Diabetes* **107,** 119–125
12. Stephens, J. M., Lee, J., and Pilch, P. F. (1997) *J. Biol. Chem.* **272,** 971–976
13. Egawa, K., Nakashima, N., Sharma, P. M., Maegawa, H., Nagai, Y., Kashiwagi, A., Kikkawa, R., and Olefsky, J. M. (2000) *Endocrinology* **141,** 1930–1935
14. Rui, L., Aguirre, V., Kim, J. K., Shulman, G. I., Lee, A., Corbould, A., Dunaif, A., and White, M. F. (20001). *J. Clin. Invest.* **107,** 181–189
15. Sun, X. J., Goldberg, J. L., Qiao, L. Y., and Mitchell, J. J. (1999) *Diabetes* **48,** 1359–1364
16. Haruta, T., Tatsuhito, U., Kawahara, J., Takano, A., Egawa, K., Sharma, P. M., Olefsky, J. M., and Kobayashi, M. (2000) *Mol. Endocrinol.* **14,** 783–794
17. Lee, A. V., Gooch, J. L., Oesterreich, S., Guler, R. L., and Yee, D. (2000) *Mol. Cell. Biol.* **20,** 1489–1496
18. Tyers, M., and Willems, A. R. (1999) *Science* **284,** 601–603
19. Palombella, V. J., Rando, O. J., Goldberg, A. L., and Maniatis, T. (1994) *Cell* **78,** 773–785
20. Kirschner, M. (1999) *Trends Cell Biol.* **9,** M42–M45
21. Ciechanover, A., and Schwartz, A. L. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 2727–2730
22. Wojcik, C. (1999) *Drug Discov. Today* **4,** 188–189
23. Winston, J. T., Strack, P., Beer-Romero, P., Chu, C. Y., Elledge, S. J., and Harper, J. W. (1999) *Genes Dev.* **13,** 270–283
24. Spencer, E., Jiang, J., and Chen, Z. J. (1999) *Genes Dev.* **13,** 284–294
25. Laney, J. D., and Hochstrasser, M. (1999) *Cell* **97,** 427–430
26. DeFronzo, R. A. (1997) *Diabetes Rev.* **5,** 177–269
27. Goldberg, A. L., Elledge, S. J., and Harper, J. W. (2001) *Sci. Am.* **284,** 68–73
28. Dennis, P. B., Fumagalli, S., and Thomas, G. (1999) *Curr. Opin. Genet. Dev.* **9,** 49–54
29. Scott, P. H., Brunn, G. J., Kohn, A. D., Roth, R. A., and Lawrence, J. C., Jr. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 7772–7777
30. Isotani, S., Hara, K., Tokunaga, C., Inoue, H., Avruch, J., and Yonezawa, K. (1999) *J. Biol. Chem.* **274,** 34493–34498
31. Nave, B. T., Ouwens, M., Withers, D. J., Alessi, D. R., and Shepherd, P. R. (1999) *Biochem. J.* **344,** 427–431
32. Sekulic, A., Hudson, C. C., Homme, J. L., Yin, P., Otterness, D. M., Karnitz, L. M., and Abraham, R. T. (2000) *Cancer Res.* **60,** 3504–3513
33. Kerouz, N. J., Horsch, D., Pons, S., and Kahn, C. R. (1997) *J. Clin. Invest.* **100,** 3164–3172
34. Shimomura, I., Matsuda, M., Hammer, R. E., Bashmakov, Y., Brown, M. S., and Goldstein, J. L. (2000) *Mol. Cell* **6,** 77–86
35. Michael, M. D., Kulkarni, R. N., Postic, C., Previs, S. F., Shulman, G. I., Magnuson, M. A., and Kahn, C. R. (2000) *Mol. Cell* **6,** 87–97
36. Merforth, S., Osmers, A., and Dahlmann, B. (1999) *Mol. Biol. Rep.* **26,** 83–87
37. Mitch, W. E., Bailey, J. L, Wang, X., Jurkovitz, C., Newby, D., and Price, S. R. (1999) *Am. J. Physiol.* **276,** C1132–C1138
38. Aguirre, V., Uchida, T., Yenush, L., Davis, R. J., and White, M. F. (2000) *J. Biol. Chem.* **275,** 9047–9054
39. Mothe, I., and Van Obberghen, E. (1996) *J. Biol. Chem.* **271,** 11222–11227
40. Hotamisligil, G. S., Peraldi, P., Budvari, A., Ellis, R. W., White, M. F., and Spiegelman, B. M. (1996) *Science* **271,** 665–668
41. Pederson, T. M., Kramer, D. L., and Rondinone, C. M. (2001) *Diabetes* **50,** 24–31