Insulin resistance is a key pathophysiologic feature of obesity and type 2 diabetes and is associated with other human diseases, including atherosclerosis, hypertension, hyperlipidemia, and polycystic ovarian disease. Yet, the specific cellular defects that cause insulin resistance are not precisely known. Insulin receptor substrate (IRS) proteins are important signaling molecules that mediate insulin action in insulin-sensitive cells. Recently, serine phosphorylation of IRS proteins has been implicated in attenuating insulin signaling and is thought to be a potential mechanism for insulin resistance. However, in vivo increased serine phosphorylation of IRS proteins in insulin-resistant animal models has not been reported before. In the present study, we have confirmed previous findings in both JCR:LA-cp and Zucker fatty rats, two genetically unrelated insulin-resistant rodent models, that an enhanced serine kinase activity in liver is associated with insulin resistance.

The enhanced serine kinase specifically phosphorylates the conserved Ser789 residue in IRS-1, which is in a sequence motif separate from the ones for MAPK, c-Jun N-terminal kinase, glycogen-synthase kinase 3 (GSK-3), Akt, phosphatidylinositol 3-kinase, or casein kinase. It is similar to the phosphorylation motif for AMP-activated protein kinase, but the serine kinase in the insulin-resistant animals was shown not to be an AMP-activated protein kinase, suggesting a potential novel serine kinase. Using a specific antibody against Ser(P)789 peptide of IRS-1, we then demonstrated for the first time a striking increase of Ser789-phosphorylated IRS-1 in livers of insulin-resistant rodent models, indicating enhanced serine kinase activity in vivo. Taken together, these data strongly suggest that unknown serine kinase phosphorylation of IRS-1 may play an important role in attenuating insulin signaling in insulin-resistant animal models.

Insulin resistance, commonly defined as a decreased ability of insulin to stimulate glucose uptake/metabolism in peripheral tissues and to inhibit hepatic glucose output, is a major pathogenic problem in many human diseases, including obesity, type 2 diabetes, atherosclerosis, hypertension, hyperlipidemia, and polycystic ovarian disease (1–3). Although knowledge of the molecular mechanism of insulin action has been greatly enhanced, the molecular basis for insulin resistance remains unknown. Insulin receptor substrate (IRS)1 proteins are key molecules of the insulin signaling cascade (4, 5). They are tyrosyl-phosphorylated upon insulin stimulation, thereby trigging intracellular signaling through recruitment of proteins with the Src homology-2 domain, including PI3K, Grb-2, Nck, fyn, and Shp-2 among others (4, 6–11). Studies of mice with a targeted disruption of IRS-1 or IRS-2 revealed insulin resistance (12–14). Consistent with this, a defect in tyrosyl phosphorylation of IRS proteins is associated with insulin resistance in human type 2 diabetes as well as in insulin-resistant animals and cultured cells (15–21), suggesting that the molecular basis for insulin resistance may reside at the level of IRS proteins. However, the specific molecular mechanism is not known. A hypothesis has emerged recently that serine/threonine phosphorylation of IRS proteins (via enhanced serine/threonine kinase activity) decreases the ability of IRS proteins to be phosphorylated on tyrosine, thereby attenuating insulin signaling (20–29). Several serine kinases have been reported to phosphorylate IRS-1 in vitro and/or in vivo, including MAPK (at Ser612), glycogen-synthase kinase 3 (GSK-3), casein kinase (at Thr283/Ser307), PI3K, mTor (at Ser663,693), c-Jun N-terminal kinase (at Ser327), and Akt (30–39) and have been implicated in various manipulations that impair insulin signaling in cultured cells. However, whether any of these kinases and serine phosphorylation of IRS-1 are associated with insulin resistance in animal models has not been established.

We previously studied liver extracts from insulin-resistant rodents and showed enhanced serine kinase activity for IRS-1 in vitro (40). In this report, we have identified the specific serine phosphorylation site (Ser789) on IRS-1 for the enhanced serine kinase activity. We then used a specific antibody that recognizes Ser(P)789 to confirm the increased Ser789 phosphorylation of IRS-1 in two genetically unrelated insulin-resistant rat models: Zucker fatty rats and JCR:LA-cp obese rats. The identity of the enhanced serine kinase is still not known; however, our study excluded all serine kinases known to phosphorylate IRS-1, indicating that a novel serine kinase exists that may underlie the molecular mechanism of insulin resistance.

The abbreviations used are: IRS, insulin receptor substrate; PI3K, phosphatidylinositol 3-kinase; TPCR, 1-tosylamido-2-phenethyl chloromethyl ketone; AMPK, AMP-activated protein kinase; MAPK, mitogen-activated protein kinase; DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride; CHO, Chinese hamster ovary cells; GST, glutathione S-transferase; BSA, bovine serum albumin; SAMS, peptide; HPMASGLHFLVRKR; HPLC, high performance liquid chromatography; TFA, trifluoroacetic acid; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; CMV, cytomembrane virus; PBS, phosphate-buffered saline; TNP, tumor necrosis factor; JNK, c-Jun N-terminal kinase; mTor, target of rapamycin; mammalian.

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EXPERIMENTAL PROCEDURES

Animals—JCR:LA-cp rats in this study were 6-month-old males, bred as previously described (kindly provided by Dr. Russell at the University of Alberta) (40). Three obese (cp/cp) and three lean male animals (+/cp or +/+ ) were used in the study. Eight obese (fa/fa) Zucker and eight lean male control (Charles River) rats were used at 10 weeks of age (41). All rats were fasted for 4 h prior to experiment. All care and the animals were in accordance with the guidelines of the National Institute of Health and subjected to prior approval by the Institutional Animal Care and Use Committee of the University of Vermont.

Preparation of Liver Extracts—Livers were rapidly minced and homogenized in lysis buffer (10 mM Tris-HCl, pH 7.4, 250 mM sucrose, 10 mM NaF, 5 mM EDTA, 1 mM DTT, 0.5 mM PMSF, 0.2 mM Na3VO4, 10 g/ml leupeptin, and 10 mM apotinin) (1 g of tissue/mL) with a Brinkmann Polytron homogenizer, followed by centrifugation at 10,000 × g for 10 min (Sorvall RC-5B). The supernatants were centrifuged at 100,000 × g for 30 min in a Beckman L8-M ultracentrifuge. The supernatants were precipitated with (NH4)2SO4 at 50% saturation, followed by centrifugation at 100,000 × g for 30 min. The (NH4)2SO4 precipitates were re-dissolved in lysis buffer followed by centrifugation at top speed in a Biofuge ( Heraeus) for 15 min. The supernatants were used as the source of kinase for the in vitro kinase assay (40).

Purification of Full-length IRS-1 from CHO IR/IRS-1 Cells—CHO cells overexpressing rat IRS-1 (wild type or Ser789 to alanine mutant) and the human insulin receptor (CHO/IR/IRS-1 or CHO/IR/IRS-1/IRS-1S789A) were grown to a 85% confluence in 10-cm dishes in F-12 medium supplemented with 12% fetal bovine serum and fasted for 16 h. Dulbecco’s modified Eagle’s medium with high glucose as previously described (40, 42). Cells were lysed in homogenization buffer (20 mM Tris-HCl, pH 7.5, 137 mM NaCl, 10 mM NaF, 1 mM MgCl2, 1 mM CaCl2, 0.2 mM sodium orthovanadate, 0.5 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10% glycerol, and 1% Nonidet P-40) and centrifuged at 13,000 rpm for 15 min in a Biofuge. The supernatant was incubated with protein A-agarose (Invitrogen) and washed with protein A-agarose (Invitrogen), and the aIRS-1 immune complex was washed three times with homogenization buffer and twice with kinase buffer before use as a substrate.

Phosphorylation of IRS-1 by Liver Extracts or Recombinant Erk2—GST-IRS-1 fusion proteins or the aIRS-1 immune complexes were phosphorylated by 10 μg of liver extract (50% (NH4)2SO4 precipitates) in vitro in a final volume of 40 μl of kinase buffer (20 mM HEPES, pH 7.4, 1 mM DTT, 10 mM MgCl2, 100 mM NaF, 1 mM CaCl2, 0.2 mM sodium orthovanadate, 0.5 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10% glycerol, and 0.5% Triton X-100) and centrifuged at 12,000 rpm for 20 min (40). Phosphorylation of GST-IRS-1 fusion proteins or the aIRS-1 immune complex by recombinant Erk2 (kindly provided by Drs. James Posada and Paul Vicki at University of Vermont) were carried out in a linked in vitro recombinant MAPK assay as described previously, and were stopped by adding 10 μl of 5% trichloroacetic acid (TCA). The kinase buffer containing 0.5 mM DTT and boiled for 5 min. Proteins were separated on 10% SDS-PAGE for GST fusion proteins or 7.5% SDS-PAGE for full-length IRS-1, respectively, stained, and destained. 32P-Labeled phosphorylated proteins were visualized by autoradiography. In some cases, protein bands were excised and counted in a scintillation counter (MINAXI Tri-CarB 4000). Unlabeled phosphorylated proteins were visualized by immunoblotting analysis.

AMPK Activity in Rat Liver Extracts—The liver extract (100 μg of proteins diluted into 500 μl of lysis buffer containing 1% Nonidet P-40) was incubated with either anti-a subunit (α1), anti-α2 subunit of AMPK (α2), or non-immune serum (NI) for 2 h followed by incubating with protein A-agarose (Invitrogen) for an additional 1 h. The immune complexes were washed with the same buffer three times and kinase buffer twice before the kinase activity was measured. AMPK activity was measured by the in vitro kinase assay using GST-IRS1-(765–816) or SAMS peptide (HMRSAMSGLHLVKRR) (43) as a substrate in the presence or absence of 200 μM AMP. Phosphorylated GST-IRS1-(765–816) was analyzed by 12% SDS-PAGE. Phosphorylated SAMS peptide was spotted on Whatman P81 filter paper. The papers were air dried and resuspended in 1% phosphoric acid, dried, and counted on a scintillation counter (MINAXI Tri-CarB 4000).

Reverse-phase HPLC Separation of Phosphopeptides—Phosphorylated proteins were extracted from SDS-PAGE gels as described previously (40, 44), dissolved in 100 μl of 50 mM ammonium bicarbonate (pH 7.6) containing 0.3 mg/ml TPCK-treated trypsin (Worthington Biochemical Corp.) or in 50 μl of 0.1 M Tris-HCl, pH 7.8, and 0.01 mM CaCl2 containing 100 μg/ml chymotrypsin (Sigma), and incubated at 37°C overnight. Digests phosphopeptides were dried in a Speedvac and redissolved in 0.055% trifluoroacetic acid (TFA). The tryptic or chymotryptic phosphopeptides were separated in a Rainin Dynamax HPLC system equipped with a Hi-Pore reverse-phase RP18 column (Bio-Rad) and eluted at a flow rate of 0.5 ml/min with 0.055% TFA modified with 2% acetonitrile–0.05% TFA. Phosphopeptides were detected with a fluorescamine detector with a β-ram on-line radioactivity detector (INSUS System) or by measuring Cerenkov radiation in 0.5-mI fractions in a scintillation counter (MINAXI Tri-CarB 4000).

Phosphopeptide Mapping by Tricine SDS-PAGE—Tryptic or chymotryptic phosphopeptides isolated from HPLC were coupled to an acrylamine-Sequenon disc as described by the manufacturer (Milligen/Biosearch). Edman degradation of immobilized peptides was carried out in cycles consisting of the following steps in each cycle: (a) coupling with phenylisothiocyanate (Pierce) at 50°C for 10 min; (b) washing the disc six times with methanol before the next cycle (10, 45).

Unlabeled phosphorylated GST fusion proteins or full-length IRS-1 were subcloned into pCMV/his expression vector at SacI and HindIII sites. The pCMV/his expression vector confers histidine resistance to CHO cells (42). CHO/IR cells were transfected with pCMV/his/IRS-1 or pCMV/his/IRS-1S789A (10 μg) by calcium phosphate-mediated transfection (42). The transfected cells were selected in 0.5 mM histidinol, 200 μg/ml kanamycin, and 1% Trion X-100 and centrifuged at 12,000 × g (Sorvall RC-5B) for 20 min. The supernatant was passed through cheesecloth and centrifuged at 140,000 × g (Beckman L8-M ultracentrifuge) for 30 min. The supernatant (4 mg of total proteins) was incubated with either aIRS-1 or IRS-1S789A followed by incubating with protein A-agarose (Invitrogen). The immune complexes were washed three times with PBS containing 1% Triton X-100 and denatured in Laemmli buffer containing 0.1 × DTT.

Immunoblotting Analysis—Proteins were separated by 7.5% (for IRS-1) or 12% (for GST fusion proteins) SDS-PAGE and transferred to nitrocellulose. The membranes were blocked overnight at 4°C with 1% milk and 1% bovine serum albumin in TBS (20 mM Tris-HCl, pH 8.0,
0.15 M NaCl), and incubated with \(\frac{1}{250}\) IRS-1 (1:400) or \(\frac{1}{250}\) IRS-1PS789 (1:400) in TBST (TBS with 0.05% Tween-20) for 1 h. The membranes were washed three times with TBST, probed with horseradish peroxidase-conjugated protein A (Calbiochem) at 1:3000 for 30 min, washed three times with TBST, and washed once with TBS. Specific proteins were visualized by using an enhanced chemiluminescence system (SuperSignal, Pierce).

In Situ Immunofluorescent Detection of Serine 789 Phosphorylation of IRS-1 in Rat Livers—Liver tissues were briefly washed in ice-cold PBS and then fixed in 4.0% paraformaldehyde/PBS for 12 h at 4 \(^\circ\)C.

After extensively washing in PBS, tissues were equilibrated in 30% sucrose/PBS overnight. Sucrose-infiltrated liver was embedded in OCT cryoembedding medium (Miles Scientific) and sectioned at 5 \(\mu\)m in a cryostat. Frozen sections were mounted onto charged microslides (Charge-Plus, Fisher Scientific), hydrated in PBS, and permeabilized 20 min in PBS with 0.1% Triton X-100. Sections were blocked in PBS supplemented with 5% normal donkey serum and 1% bovine serum albumin (BSA) for 1 h at room temperature and incubated for 12 h at 4 \(^\circ\)C with \(\frac{1}{250}\) IRS1 (1:1000 dilution), \(\frac{1}{250}\) IRS1PS789 (1:1000 dilution), or their corresponding pre-immune serum diluted in PBS containing 0.1% Triton and 1% BSA. Following three washes, sections were incubated with the secondary antibody (ML-grade donkey anti-rabbit IgG-CY3, Jackson ImmunoResearch, 1:2000). Nuclei were counterstained for reference with Yo-Pro1 (Molecular Probes), which was added to the diluted secondary antibody solution at 0.5 \(\mu\)g/ml. After washing, liver sections were mounted in Aqua-PolyMount (Polysciences).

Semiquantitative Assessment of IRS-1 and IRS-1PS789 in Hepatocytes in Situ—A comparative semiquantitative assessment of both total IRS-1 and IRS-1PS789 immunoreactivity was accomplished by batch staining and sampling by confocal microscopy. Samples were imaged
with a laser-scanning confocal microscope (Bio-Rad MRC 1024) (University of Vermont College of Medicine Cell Imaging Facility) using a 60× PlanApo objective lens (numerical aperture = 1.4) and the 568-nm excitation line of an argon/krypton laser. For each field the microscope was focused to maximize the number of cells optically sectioned through the middle of the nucleus. All confocal imaging parameters were identical for each imaged field and a minimum of four non-overlapping fields (512 × 512 pixels) were transferred to a Power Macintosh G3 computer running IMAGE (version 1.62, National Institutes of Health) for image analysis.

Individual hepatocytes suitable for quantitation were identified and numbered. For each cell studied, an 18-pixel diameter circle (256 pixels total), the width of which is smaller than the cytoplasmic area between the surface and nucleus at this magnification, was used to measure mean pixel intensities (range = 0–255 grayscale levels) of the corresponding immunofluorescence signal. For uniformity in sampling, only a single area per cell was recorded, and only cells with their maximum widths in the confocal section were scored. Between 34 and 70 cells for each animal and antibody staining were analyzed and imaged under identical conditions. Field background fluorescence values (where there was no tissue) were subtracted from intensity values for each area analyzed.

Mean intensity values for an antibody staining were then corrected for nonspecific background staining by subtracting the mean fluorescence intensities of corresponding pre-immune serum. Thus, IRS-1 and IRS-1(526–859) immunoreactivities were expressed as corrected mean pixel intensities for each animal.

**RESULTS**

**Enhanced Serine Kinase Activity in Liver Extracts of Zucker Fatty Rats—** JCR:LA-cp rats and Zucker fatty rats are two genetically unrelated, widely used animal models of insulin resistance and obesity (41, 49–52). We had previously identified enhanced serine kinase activity in liver extracts from JCR:LA-cp obese rats (40). We now report the same finding in Zucker fatty (fa/fa) rats. Kinase activity in liver extracts from Zucker rats was compared with JCR:LA-cp rats using our in vitro kinase assay based on 50% (NH₄)₂SO₄ precipitates of liver extracts as the source of kinase and a GST fusion protein containing the 526- to 859-amino acid region of IRS-1 (GST-IRS-1(526–859)) as substrate (40). Serine kinase activity was 3- and 2.5-fold higher in the Zucker fatty rats and JCR:LA-cp obese rats, respectively, compared with their lean controls (Fig. 1A).

To see if the same site within IRS-1 was serine-phosphorylated in Zucker and JCR:LA-cp rats, phosphorylated GST-IRS1-(526–859) was digested with trypsin and the tryptic phosphopeptides were analyzed by Tricine SDS-PAGE and HPLC analyses. An identical tryptic phosphopeptide (P3) was identified by Tricine SDS-PAGE analysis in samples phosphorylated by liver extracts from both JCR:LA-cp and Zucker rats, with greatly increased density in the obese rats over their lean controls (Fig. 1B, lanes a–d). When GST-IRS1-(526–859) was phosphorylated by Erk-2, P1 and P2 were the predominant tryptic phosphopeptides, and P3 was not detected (Fig. 1B, lane e). HPLC analysis showed a single tryptic phosphopeptide (eluted at 12.8 min) phosphorylated by the JCR:LA-cp rats (Fig. 1C, top panel) that was ~2-fold increased in the obese rats over the lean controls. This phosphopeptide was P3, because it co-migrated with P3 on Tricine SDS-PAGE (data not shown). An identical HPLC elution profile was obtained from tryptic peptide phosphorylated by extracts from the Zucker rats (Fig. 1C, middle panel). In contrast, the phosphorylation pattern of GST-IRS1(526–859) generated by recombinant Erk-2 was totally different with multiple tryptic phosphopeptides, none of which eluted at 12.8 min (Fig. 1C, bottom panel). These data indicate that the enhanced serine kinase activity in both insulin-resistant models phosphorylates IRS-1 at seemingly identical serine residues, which are distinctly different from the ones phosphorylated by Erk-2 kinase, confirming that the responsible kinase in these insulin-resistant animals is not an MAPK (40).

To see if other regions of IRS-1 are serine-phosphorylated by the enhanced serine kinase activity, we performed a similar experiment on full-length recombinant IRS-1 protein that was prepared from CHO/IR/IRS-1. As expected, increased phosphorylation of IRS-1 was detected by in vitro kinase assay using extracts from both the obese JCR:LA-cp and Zucker rats (Fig. 2A, lanes b and d versus lanes a and c). Tryptic phosphopeptide mapping analyzed by Tricine SDS-PAGE showed that P3 was the major tryptic phosphopeptide that increased in both the obese JCR:LA-cp and the Zucker fatty rats (Fig. 2B, lanes b and d versus lanes a and c), suggesting the phosphorylation site in the full-length IRS-1 and GST-IRS1(526–859) was identical. A few minor additional phosphopeptides were found when using extracts from the Zucker rats; however, there was no significant difference between the lean and fatty rats (Fig. 2B, lanes c and d). Recombinant Erk-2 phosphorylated the full-length IRS-1 mainly on P1 and P2 tryptic peptides with an additional few weak phosphopeptides; however, P3 tryptic peptide was not detected (Fig. 2B, lane e). These data confirm that P3 is the only tryptic phosphopeptide in IRS-1 induced by the enhanced serine kinase activity of the insulin-resistant rats.

**Determination of the Specific Serine Phosphorylation Site for Enhanced Serine Kinase Activity—** To determine the specific serine phosphorylation site(s), the tryptic phosphopeptide P3 was purified by HPLC (Fig. 3A, left panel) followed by manual radiosequencing analysis based on Edman degradation (10, 45). The radioactivity was released at the fourth cycle of Edman degradation, indicating the phosphorylated serine was

**FIG. 2.** Phosphorylation of full-length IRS-1 by the serine kinase activity of liver extracts and recombinant Erk-2. A, IRS-1 was purified from CHO/IR/IRS-1 cells by immunoprecipitation with anti-IRS-1 antibody and phosphorylated by 50% (NH₄)₂SO₄ precipitates of liver extracts or recombinant Erk-2 in an in vitro kinase assay. Phosphorylated proteins were separated on a 7.5% SDS-PAGE gel and visualized by autoradiography. B, phosphorylated IRS-1 was extracted from gels, digested with trypsin, and separated by 16% Tricine SDS-PAGE. Phosphopeptides were visualized by autoradiography. Major phosphopeptides P1, P2, and P3 are marked on the left.
at the fourth position (Fig. 3A, right panel). Trypsin cleaves peptide bonds at the C-terminal of lysine or arginine except between proline and lysine or arginine (53). Serine 789 was the only candidate in GST-IRS1-(526–859) to be a fourth residue from a trypptic cleavage site (Fig. 3A, right panel). Having noticed that the −3 position of Ser^{789} is a leucine residue that can be cleaved at the C terminus by chymotrypsin (53), phosphorylated GST-IRS1-(526–859) was digested with chymotrypsin followed by HPLC separation. A single phosphopeptide was isolated (Fig. 3B, left panel), and, as expected, radioactivity was detected at the third position by manual radioscanning analysis (Fig. 3B, right panel). Tryptic and chymotryptic phosphopeptides phosphorylated by extracts from JCR:LA-cp rats were analyzed in an identical fashion, and similar results were obtained (data not shown).

To further confirm that the phosphorylation site was Ser^{789}, two additional GST fusion proteins were generated: one containing amino acids 765–816 of IRS-1 (GST-IRS1-(765–816)) and another containing IRS-1-(526–859) with Ser^{789} mutated to glycine (GST-IRS1-(526–859)/Ser^{789}G) (Fig. 4A). These GST fusion proteins were exposed to 50% (NH₄)₂SO₄ precipitates of liver extracts (Fig. 4B, lane a) versus lanes b–d). Failure of AMP to phosphorylate Ser^{789} residue in IRS-1 is the phosphorylation site for the enhanced serine kinase activity identified in insulin-resistant animals, and the serine kinase is not an MAPK.

Enhanced Serine Kinase Activity Is Not Contributed by an AMP-activated Protein Kinase—The serine 789 phosphorylation site in IRS-1 is highly conserved among human, rat, and mouse (Fig. 5). It is surrounded by leucines at the P − 5 and P + 4 positions, which resemble a phosphorylation motif for AMP-activated protein kinase (43, 54). AMP-activated protein kinase (AMPK) is a heterotrimer of three subunits, i.e. α, β, and γ. The 63-kDa α-subunit contains the kinase domain, and two isoforms of the α-subunits (α1 and α2) have been described (55). AMP binds to the α-subunit of AMPK and increases its kinase activity by 5- to 8-fold (56). Recently, AMPK has been reported to phosphorylate Ser^{789} of IRS-1 (39). To see if the enhanced serine kinase activity detected in the insulin-resistant rats is derived from AMPK, we performed the in vitro kinase assay in the presence or absence of AMP using GST-IRS1-(765–816) as a substrate. As previously noted, kinase activity in liver extracts was higher in obese JCR:LA-cp rats than in lean control rats in the absence of AMP. In the presence of AMP, the kinase activity did not increase in either the lean or the obese tissue extract (Fig. 6A). Failure of AMP to activate serine kinase activity was also observed in the extracts prepared from Zucker rats as well as from insulin-treated CHO cells (data not shown). To see if depletion of AMPK by specific antibodies will alter the enhanced serine kinase activity in liver extracts, AMPK was immunoprecipitated from liver extracts by anti-α1, anti-α2 antibodies or non-immune serum, and kinase activities in both supernatant and the immune complex were measured. In the supernatants after immunodepletion of AMPK, there was slightly decreased kinase activity in lean animals (Fig. 6B, upper panel, lane a versus lanes b and c); however, the en-
r IRS-1: \( 780^{RQHLRLSSSGRLRTATAEDSS}{803} \)

mIRS-1: \( 793^{RQHLRLSSSGRLRTATAEDSS}{818} \)

h IRS-1: \( 785^{RQHLRLSTSSGRLLYATADD}{808} \)

**Fig. 5.** Alignment of corresponding amino acid sequence around Ser\(^{789}\) for rat, mouse, and human IRS-1. The identified phosphorylation site is indicated by an arrow.

Enhanced serine kinase activity in obese animal was not affected (Fig. 6B, upper panel, lanes e and f versus lane d). Consistent with Fig. 6A, AMP failed to activate kinase activities in the supernatants (Fig. 6B, upper panel, lanes g–l versus a–f). In contrast to the supernatant, AMP-activated kinase activities were readily detected in both anti-\(\alpha\) and anti-\(\alpha2\) immune complexes whether using GST-IRS1-(765–816) as a substrate (Fig. 6B, lower panel) or SAM (specific AMPK substrate) (Fig. 6C). However, there was no difference between lean and obese animals (Fig. 6, B, lower panel, and C). The fact that AMPK activities can only be detected in immune complexes but not in tissue extracts (Fig. 6A) indicates that AMPK activity was suppressed in liver extracts. Together, these data suggest that the enhanced serine kinase activity in insulin-resistant animals is not AMP-activated kinase, indicating a novel serine kinase.

**In Vivo Detection of Serine 789 Phosphorylation of IRS-1 in Liver Tissues**—The fact that enhanced serine kinase phosphorylates IRS-1 in vitro does not necessarily mean it occurs in vivo. To confirm that enhanced serine kinase phosphorylates IRS-1 in vivo, we generated an antibody against a synthetic peptide of IRS-1 that contained Ser\(^{789}\) (\(\alpha\)IRS1\(^{PST89}\)). The specificity of the antibody was tested by immunoblotting against GST-IRS1-(765–816), which had been exposed to the liver extracts in the presence (phosphorylated) or absence of ATP (unphosphorylated) in the in vitro kinase assay. Although there was 0.5 \(\mu\)g of GST-IRS1-(765–816) protein in each lane, GST-IRS1-(765–816) was detected by \(\alpha\)IRS1\(^{PST89}\) only if ATP was present in the in vitro kinase assay (Fig. 7A, lanes e, d, g, and h versus lanes a, b, e, and f), indicating the specificity of the antibody against phosphorylated GST-IRS1-(765–816). Because IRS-1 has been shown to be heavily phosphorylated at multiple serine sites in vivo in the basal state (42), we tested whether \(\alpha\)IRS1\(^{PST89}\) recognizes serine phosphorylation sites other than Ser\(^{789}\). This was done by immunoblotting full-length IRS-1 or Ser\(^{789}\) to alanine mutant (IRS-1\(^{S789A}\)) isolated from CHO/IR cells overexpressing wild type (CHO/IR/IRS-1) or IRS-1\(^{S789A}\) (CHO/IR/IRS-1\(^{S789A}\)), respectively. After exposed to the liver extracts by in vitro kinase assay, wild type, but not IRS-1\(^{S789A}\), was recognized by \(\alpha\)IRS1\(^{PST89}\) (Fig. 7B, lanes a–d versus f–g). Although both substrates were phosphorylated by Erk-2 equally well (Figs. 2A, lane e, and 4B, panels I and II, lane e), they were not recognized by \(\alpha\)IRS1\(^{PST89}\) (Fig. 7B, lanes e and h). Thus, \(\alpha\)IRS1\(^{PST89}\) is specific for Ser\(^{789}\) of IRS-1 and does not recognize phosphorylated IRS-1 at serines other than Ser\(^{789}\).

We used this highly specific antibody to investigate liver sections from the lean and obese Zucker rats. Total IRS-1 and Ser\(^{789}\)-phosphorylated IRS-1 were analyzed in situ by immunofluorescence staining of liver sections from 10-week-old Zucker fatty rats and lean controls (n = 2 each group). Utilizing large batch staining and sampling analysis with identical confocal imaging parameters for each sample, we observed a decreased total IRS-1 immunofluorescence signal but an increased Ser\(^{789}\) phosphorylation signal in the obese rat liver when compared with their lean controls (Fig. 8A, panels a–d). Semiquantitative analyses of IRS-1 and IRS-1\(^{PST89}\) immunofluorescence signals consistently revealed a 76% decreased
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IRS-1 immunoreactivity and 247% increased IRS-1\(^{PST89}\) immunoreactivity in the obese group versus lean controls (Fig. 5B). Similar results were obtained when JCR:LA-cp rats were examined (data not shown).

IRS-1 total protein or Ser\(^{789}\)-phosphorylated IRS-1 were also immunoprecipitated from liver lysates of Zucker fatty rats and lean controls (n = 3 each group) by αIRS-1 or αIRS\(^{PST89}\), followed by immunoblotting analysis with the same antibodies. In Zucker fatty rats, there was a lower level of total IRS-1 (58% of lean control) (Fig. 9, A, lanes b, d, and f, versus lanes a, c, and e, and C). In contrast, Ser\(^{789}\) phosphorylation of IRS-1 as detected by αIRS\(^{PST89}\) was significantly increased in the liver of Zucker fatty rats (191%) opposed to the lean controls (Fig. 9, B, lanes b, d, and f, versus lanes a, c, and e, and C). Together with the immunofluorescent staining, these data demonstrate, for the first time, that Ser\(^{789}\) phosphorylation of IRS-1 is significantly increased in the liver of insulin-resistant rat models.

**DISCUSSION**

Serine phosphorylation of IRS-1 has been implicated as a mechanism of attenuated insulin signaling, the so-called “insulin resistance” (20–29, 37). Identification of serine phosphorylation sites on IRS-1 and the kinase that phosphorylates IRS-1 are important steps toward the understanding of the molecular mechanism of insulin resistance. We previously identified enhanced serine kinase activity in liver extracts of JCR:LA-cp obese rats using an in vitro kinase assay combined with phosphotryptic peptide mapping analysis (40). The current study identified that the phosphorylation site for this kinase is Ser\(^{789}\) of IRS-1. Furthermore, we now demonstrate in vivo for the first time that Ser\(^{789}\) phosphorylation of IRS-1 is increased in liver of the insulin-resistant rat model using both immunofluorescence staining or immunoblotting analysis. The fact that identical results were obtained in two genetically unrelated insulin-resistant rat models, JCR:LA-cp and Zucker rats (41, 49), suggests that our findings may generally apply to the insulin-resistant state. Conclusive evidence of increased Ser\(^{789}\) phosphorylation of IRS-1 in vivo together with the in vitro detection of enhanced serine kinase activity in both insulin-resistant animal models leads us to speculate that this as yet unidentified serine kinase, which physiologically or pathophysiologically modulates insulin sensitivity, is a mechanism of impaired insulin effectiveness in liver.

IRS proteins are important signaling molecules that become tyrosyl-phosphorylated during insulin stimulation and activate the insulin signaling network through interaction with downstream signaling molecules, including PI3K, Grb-2/Sos, and SHP-2 (5, 57). Recent studies in cultured cells have shown that IRS-1 becomes serine-phosphorylated after prolonged exposure to many factors, including insulin, TNF-α, glucose, free fatty acids, and consequently fails to become tyrosyl-phosphorylated, resulting in attenuation of the insulin response (20–29, 37). Based on these findings, an attractive hypothesis has emerged that serine phosphorylation of IRS proteins is a cause of insulin resistance. Searching for IRS-1 serine kinases led to the identification of many serine kinases, including casein kinase II, P13K, Akt and mTor (33, 37, 38, 58). Glycogen-synthase kinase 3 and MAPK have also been reported to phosphorylate IRS-1 in

**Fig. 6. Enhanced serine kinase activity is not AMP-activated protein kinase.** A, GST-IRS1-(765–816) was phosphorylated by liver extracts (JCR:LA-cp rats) in the in vitro kinase assay in the absence or presence of 200 μM AMP. B, AMPK were immunoprecipitated from liver extracts with anti-α1, anti-α2, or non-immune serum (NI). Kinase activity in the supernatants (upper panel) and immune complexes (lower panel) were measured by the in vitro kinase assay using GST-IRS1-(765–816) as a substrate. C, kinase activity in the immune complexes was measured by the in vitro kinase assay using SAMS peptide as a substrate in the presence or absence of 200 μM AMP.

**Fig. 7. Characterization of αIRS1\(^{PST89}\) specificity for Ser(P)\(^{789}\) of IRS-1.** A, GST-IRS1-(765–816) was phosphorylated by 50% (NH₄)₂SO₄ precipitates of liver extracts from JCR:LA-cp and Zucker rats in the in vitro kinase assay in the presence or absence of ATP. B, wild type and IRS-1\(^{PST89}\) proteins were isolated from CHOIR/IRS-1 and CHOIR/IRS-1\(^{PST89}\) cells by immunoprecipitation with αIRS-1. The immune complexes were phosphorylated by 50% (NH₄)₂SO₄ precipitates of liver extracts from JCR:LA-cp or Zucker rats or by Erk-2 in the in vitro kinase assay. Proteins from A and B were then separated on 10% SDS-PAGE gels (for GST-IRS1-(765–816)) or 7.5% SDS-PAGE gels (for full-length IRS-1), transferred to nitrocellulose membranes and immunoblotted with αIRS1\(^{PST89}\) or αIRS1 as indicated.
and imaged under identical conditions (see each animal were analyzed with laser-scanning confocal microscopy with the corresponding pre-immune serum. Between 34 and 70 cells for cytocytes stained with immune serum corrected for background staining. Values represent the mean cytoplasmic fluorescence intensity of hepatocytes from liver sections from lean (a) and obese (b and d) rats stained with aIRS-1 (red) (a and b) or aIRS1PS789 (red) (c and d) by indirect immunofluorescence. Nuclei were counterstained green for reference. B, semiquantitative assessment of aIRS-1 and aIRS1PS789 immunoreactivity in hepatocytes from liver sections from Zucker lean and obese rats. Relative aIRS-1 immunoreactivity and aIRS1PS789 immunoreactivity were the mean ± S.E. from three lean and three obese rats. Values represent the mean cytoplasmic fluorescence intensity of hepatocytes stained with immune serum corrected for background staining with the corresponding pre-immune serum. Between 34 and 70 cells for each animal were analyzed with laser-scanning confocal microscopy and imaged under identical conditions (see “Experimental Procedures” for details).

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We thus took an alternate approach to investigate serine phosphorylation of IRS-1 using tissue extracts from insulin-resistant rodents. Most importantly, we used two genetically distinct models, Zucker fatty (fa/fa) rats and JCR:LA-cp rats (41, 49–52, 60). We established an in vitro kinase assay using GST-IRS-1 fragments as substrates and tissue extracts as the kinase source (40) and identified an enhanced serine kinase activity, in liver extracts from both obese animals, that phosphorylates IRS-1 at a region between amino acid residues 526 and 859. The current study has determined the Ser789 residue in IRS-1 as the phosphorylation site for this serine kinase based on several criteria. First, radioamino acid sequencing analysis data predicted that Ser789 was the phosphorylation site. Second, GST-IRS1-(765–816) eliminated all the MAPK phosphorylation sites without altering phosphorylation by the liver extracts, but mutation on Ser 789 of IRS-1 completely abolished phosphorylation by the enhanced serine kinase source (40) and identified an enhanced serine kinase activity. Furthermore, Ser789 appears to be the only major phosphorylation site in IRS-1 for the enhanced serine kinase activity, because full-length IRS-1 produced one predominant tryptic phosphopeptide (P3) that was identical to that derived from phosphorylated GST-IRS1-(526–859) or GST-IRS1-(765–816) on Tricine SDS-PAGE and HPLC profiles.

The previously cited studies have shown that various agents (TNF-α, insulin, and glucose) promote serine phosphorylation of IRS-1, but there has been no in vivo evidence prior to this study supporting the validity of these findings to the insulin resistance in animal models. Mapping the serine phosphorylation site in IRS-1 to Ser789 allowed us to generate a phosphoserine-specific antibody (aIRS1PS789) for in vivo testing. The specificity of aIRS1PS789 was confirmed by several criteria. First, aIRS1PS789 did not recognize GST-IRS1-(765–816) protein even in a high concentration (0.5 μg/lane) by immunoblotting unless it was phosphorylated by liver extracts in vitro in the presence of ATP. Second, aIRS1PS789 recognized wild type IRS-1 by immunoblotting when phosphorylated in vitro by the liver extracts, but mutation on Ser789 of IRS-1 completely eliminated this recognition. Because both wild type IRS-1 and IRS-185789A in CHO/IR/IRS-1 or CHO/IR/IRS-185789A cells are heavily phosphorylated on serines in the basal state (manifested by the identical high molecular mass of 185 kDa on SDS-PAGE gel) (40, 42), our data show that aIRS1PS789 is highly specific for Ser(P)789 and does not recognize other phosphoserines in IRS-1.

aIRS1PS789 was subsequently used to assess the Ser789 phosphorylation of IRS-1 in vivo using liver slices from normally sensitive and insulin-resistant Zucker rats. Using semiquanti-
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tative immunofluorescence imaging, we clearly demonstrated that Ser789 phosphorylation of IRS-1 markedly increased in livers of the two studied insulin-resistant animal models. Increased Ser789 phosphorylation of IRS-1 in insulin-resistant animals was further confirmed by immunoprecipitation and immunoblotting analysis. This finding is dramatic, because the level of IRS-1 protein was decreased in the insulin-resistant obese rats as reported from other laboratories (15, 18). Increased Ser789 phosphorylation of IRS-1 in vivo in insulin-resistant states leads us to speculate that the enhanced serine kinase activity could be the potential molecular linker for insulin resistance in livers of these animals.

The identity of the responsible serine kinase is currently unknown. Serine 789 exists in a highly conserved motif among human, rat, and mouse IRS-1 (Fig. 5) and is not part of a known motif for MAPK, JNK, GST-3, casein kinase II, PI3K, Akt, or mTor. Interestingly, this motif is similar to one of the phosphorylation motifs for AMP-activated kinase: XXXXXSXXXY, where Y is a hydrophobic residue (Met, Val, Leu, Ile, or Phe) (43, 54). The fact that AMPK is able to phosphorylate IRS-1 at Y suggests that AMPK may be the AMP-activated kinase that phosphorylates IRS-1 and is not part of a known insulin resistance in livers of these animals. The definitive identification will depend on the purification and characterization of this serine kinase.

MAPK has been shown to phosphorylate IRS-1 in vitro and in vivo at Ser612, leading to the inhibition of subsequent tyrosine phosphorylation by the insulin receptor (30, 31, 61). Moreover, the phosphorylation of a synthetic IRS-1 peptide containing Ser612 by liver extracts was significantly higher in ob/ob mice than in lean controls (30). We did not detect enhanced MAPK activity in liver extracts from either the JCR-LA-cp obese rats or Zucker fatty rats, even when myelin basic protein from the low density microsomes and subsequent degradation of IRS proteins, resulting in a dissociation of IRS proteins (70, 71). It is possible that phosphorylation of Ser789 links IRS-1 to the ubiquitin-proteasome degradation pathway. This possibility is currently under investigation.

In summary, our results demonstrated in vitro and in vivo for the first time that a novel serine kinase activity assessed by Ser789 phosphorylation of IRS-1 closely correlates with the insulin-resistant state in two genetically unrelated insulin-resistant animal models. We speculate that this potential serine kinase may physiologically or pathophysiologically modulate levels of phosphorylation of IRS-1 at Ser789 and thus the insulin sensitivity.

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In Vivo Phosphorylation of Insulin Receptor Substrate 1 at Serine 789 by a Novel Serine Kinase in Insulin-resistant Rodents

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