Identification of Enhanced Serine Kinase Activity in Insulin Resistance*

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Insulin receptor substrate (IRS) proteins play a crucial role as signaling molecules in insulin action. Serine phosphorylation of IRS proteins has been hypothesized as a cause of attenuating insulin signaling. The current study investigated serine kinase activity toward IRS-1 in several models of insulin resistance. An in vitro kinase assay was developed that used partially purified cell lysates as a kinase and glutathione S-transferase fusion proteins that contained various of IRS-1 fragments as substrates. Elevated serine kinase activity was detected in Chinese hamster ovary/insulin receptor (IR)/IRS-1 cells and 3T3-L1 adipocytes chronically treated with insulin, and in liver and muscle of obese JCR:LA-cp rats. It phosphorylated the 526–859 amino acid region of IRS-1, whereas phosphorylation of the 2–516 and 900–1235 amino acid regions was not altered. Phosphopeptide mapping of the 526–859 region of IRS-1 showed three major phosphopeptides (P1, P2, and P3) with different patterns of phosphorylation depending on the source of serine kinase activity. P1 and P2 were strongly phosphorylated when the kinase activity was prepared from insulin-resistant Chinese hamster ovary/IR/IRS-1 cells, weakly phosphorylated by the kinase activity from insulin-resistant 3T3-L1 adipocytes, and barely phosphorylated when the extract was derived from insulin-resistant liver. In contrast, P3 was phosphorylated by the serine kinase activity prepared from all insulin-resistant cells and tissues of animals. P1 and P2 phosphorylation can be explained by mitogen-activated protein kinase activity based on the phosphopeptide map generated by recombinant ERK2. In contrast, mitogen-activated protein kinase failed to phosphorylate the P3 peptide, suggesting that another serine kinase regulates this modification of IRS-1 in insulin-resistant state.

An early event in insulin action, after receptor binding, is activation of tyrosine kinase activity in the cytosolic domain of the receptor (1). Insulin receptor substrate (IRS) proteins are physiological substrates for insulin receptor tyrosine kinase (2–5), and tyrosyl-phosphorylated IRS proteins serve as docking proteins by providing binding sites for SH2 domain containing proteins, including PI 3-kinase, Grb2/Sos, SHP2, c-Fyn, and Nck (2, 3, 6–8). These events lead to activation of multiple signaling pathways that are required for insulin pleiotropic action (6, 9). The importance of IRS proteins in insulin action has been demonstrated by gene disruption experiment. A lack of IRS-1 or IRS-2 in mice results in hyperinsulinemia and insulin resistance (10–13), with disruption of IRS-2 also impairing pancreatic β-cell function, so that diabetes occurs (13). Thus, IRS proteins are indispensable for normal insulin action.

IRS-1 and IRS-2 contain more than 30 serine/threonine residues in consensus sequences for many serine/threonine kinases, including casein kinase II, cAMP-dependent protein kinase, protein kinase C, cdc2 kinase, MAP kinase, and Akt/protein kinase B (2, 3, 14, 15). Recently, serine/threonine phosphorylation of IRS-1 has been linked to attenuation of insulin signaling in a cell culture system. For instance, okadaic acid is a potent and specific inhibitor for type 1 and 2A protein phosphatase (16–18). It induces hyper-serine/threonine phosphorylation of IRS-1 and impairs insulin-induced tyrosine phosphorylation of IRS-1, PI 3-kinase activation, and glucose uptake in 3T3-L1 adipocytes (16, 19, 20). Moreover, chronically treating cells with insulin or tumor necrosis factor-α induces serine/threonine phosphorylation of IRS-1 and impairs insulin action (21–25). In vitro studies have shown that serine phosphorylation of IRS-1 lowers its tyrosine phosphorylation by the insulin receptor. After dephosphorylation, IRS-1 regains its ability to be tyrosyl-phosphorylated (25). Furthermore, serine/threonine phosphorylation of IRS-1 is 4-fold increased in cardiomyocytes of obese rats and was suggested to play a role in the pathogenesis of the insulin resistance in these animals (26).

The search for serine/threonine kinases has led to the identification of several candidates based on their ability to phosphorylate IRS-1 in vitro and in vivo. Casein kinase 2 phosphorylates rat IRS-1 mainly on Thr502, and weakly on Ser99; however, the major site (Thr502) is not conserved between human and rat (27). PI 3-kinase possesses dual kinase activity that phosphorylates inositol lipids at the D-3 position of the inositol ring and phosphorylates IRS-1 and the p85 regulatory subunit of PI 3-kinase on serine sites (28–33). More recently, glyogen synthase kinase 3 and ERK2 have been found to phosphorylate IRS-1 in vitro and in vivo, converting IRS-1 into an inhibitor of insulin receptor tyrosine kinase (34–36). Despite all of these observations, the physiological relevance of these serine kinases to insulin resistance has not been established.

The JCR:LA-cp rat, when homozygous for the corpulent gene (cp/cp), is obese, hyperphagic, and hyperlipidemic (37). It dephosphorylates insulin receptor; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; DTT, dithiothreitol; Tricine, N-tris(hydroxymethyl)-methylglycine; TBS, Tris-buffered saline.
develops a profound insulin resistance between 4 and 7 months of age with hyperinsulinemia and impaired glucose tolerance, although fasting plasma glucose is normal (38). Rats with normal homozygous (+/+ ) or heterozygous (cp/cp) are lean and metabolically normal. The JCR:LA-cp rat has been used as a model to study the metabolic and pathophysiological aspect of insulin resistance (38, 39).

This study was aimed at defining serine/threonine kinase activity in chronic insulin-treated cells and obese JCR:LA-cp rats based on an in vitro kinase assay, which used GST-IRS-1 fragments as substrates and partially purified cell or tissue extracts as the source of kinase activity. Elevated serine kinase activity was detected both in the insulin-resistant culture cells, and in livers and muscles from obese JCR:LA-cp rats. Phosphorylation of IRS-1 was located exclusively in the 526–859 amino acid region. Phosphoprotein mapping demonstrated that this serine kinase activity was not related to MAP kinase activity, suggesting that it is most likely attributable to a unique and yet to be identified IRS-1 serine kinase.

**EXPERIMENTAL PROCEDURES**

**Cell Culture—**CHO cells overexpressing the human insulin receptor and rat IRS-1 (CHO/HIR/IRS-1) (40) were grown in Ham's F-12 medium supplemented with 10% fetal bovine serum (FBS). Twelve h before each experiment, the culture medium was changed to serum-free Dulbecco's modified Eagle's medium with high glucose (DMEM/high glucose) (40). 32D cells overexpressing the human insulin receptor and rat IRS-1 (32D/HIR/IRS-1) or the human insulin receptor and mouse IRS-2 (32D/ IR/IRS-2) were grown in RPMI 1640 medium supplemented with 10% FBS and 5% WEHI-3B conditional medium (3, 41). Both CHO and 32D cells were cultured at 37 °C in a humidified atmosphere composed of 95% air and 5% CO₂. 3T3-L1 cells were maintained as fibroblasts in DMEM/high glucose containing 10% calf serum and 5% glucose (42).

**Animals—**JCR:LA-cp rats in this study were males, bred as described previously (43). The rats were individually housed and fed with standard rat chow. The lights were maintained on a reversed cycle, with lights on at 8:00 p.m. and off at 8:00 a.m. Two obese (cp/cp) and two lean male animals (represented as +/+ at breed as 2:1 mixture of +/cp and +/+ ) were used in this study initially. They were all 6 months of age, with fasting blood glucose, triglyceride, and insulin levels of 210 ± 1 mg/dl, 56 ± 1 mg/dl and 45 ± 5 microunits/ml for lean and 201 ± 5 mg/dl, 106 ± 8 mg/dl, and 106 ± 6 microunits/ml for obese rats, respectively. All care and treatment of the animals was in accordance with guidelines of the Canadian Council on Animal Care and the National Institutes of Health. The protocols were approved by the Institutional Animal Care and Use Committees of the University of Alberta and the University of Vermont.

**GST Fusion Proteins—**The N-terminal (amino acids 2–516, designated as IRS-1N), middle (amino acids 526–859, IRS-1M) and C-terminal (amino acids 900–1235, IRS-1C) portions of rat IRS-1 protein, and N-terminal (amino acids 526–693, designated as N-IRS-1N) and C-terminal (amino acids 721–859, C-IRS-1M) of IRS-1 were expressed as glutathione S-transferase (GST) fusion proteins with the pGEX-2T vector (Amersham Pharmacia Biotech) (44). The DNAs encoding these regions of IRS-1 were synthesized by the polymerase chain reaction, using a rat IRS-1 cDNA as a template and pairs of oligonucleotide primers that contain appropriate restriction sites bordering these fragments (8). The PCR products were isolated, digested with appropriate restriction enzymes and subcloned into pGEX-2T, which were used to transform Escherichia coli DH5α. Transformed cells were grown to an A₆₀₀nm of 0.8 in LB medium supplemented with 0.1 mg/ml ampicillin and 25 µg/ml 3-thiol glutathione (BioStart). Fusion proteins were purified by affinity chromatography on glutathione-Sepharose column (Amersham Pharmacia Biotech) and eluted by glutathione as described by Smith and Johnson (44). Glutathione was removed by dialysis against phosphate-buffered saline (PBS) containing 10 µM dithiothreitol (DTT). All GST fusion proteins had the expected molecular weight when analyzed by SDS-PAGE.

**Preparation of Cell and Tissue Extracts—**Cells were treated with insulin (100 nM) for the indicated liquid times, and then filtered in liquid nitrogen and thawed in cold lysate buffer (10 mM Tris-HCl, pH 7.4, 250 mM sucrose, 100 mM NaF, 5 mM EDTA, 5 mM EGTA, 1 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM NaN₃, 10 µg/ml leupeptin (Sigma), 0.3 mg/ml L-1-tosylamido-2-phenylethyl chloromethyl ketone trypsin inhibitor (Sigma), and 0.025% sodium azide). Cells were also pretreated with 50 µM PD98059 (New England Biolabs) for 1 h before insulin treatment. Livers and muscles from male JCR:LA-cp obese (cp/cp) and lean (+/+ ) rats were rapidly minced and homogenized in lysis buffer (1 g of tissue/10 ml with a Brinkman homogenizer, followed by centrifugation at 10,000 × g for 10 min (Sorval RC-5B). The supernatants were centrifuged at 100,000 × g for 30 min in Beckman L-8 M ultracentrifuge. The supernatants were sequentially precipitated with (NH₄)₂SO₄ at 30, 50, and 90% saturation, followed by centrifugation at 100,000 × g for 30 min for each precipitation. (NH₄)₂SO₄ precipitates were redissolved in lysis buffer followed by centrifugation at top speed in a Biofuge (Heraeus) for 15 min. The recovered supernatants were desalted and used as the source of kinase for the in vitro kinase assay.

**Phosphorylation of IRS-1 Fusion Proteins by Cell Lysates—**The in vitro kinase assay was carried out in a final volume of 40 µl of kinase buffer (20 mM HEPES, pH 7.4, 1 mM DTT, 10 mM MgCl₂, 100 µg/ml bovine serum albumin, 0.5 µg/ml okadaic acid (Sigma), 50 µM cold ATP, 5 µCi of [γ-32P]ATP) containing 10 µg of substrate and 10 µg of protein kinase inhibitor (Promega), myristoylated protein kinase C peptide inhibitor (Promega), or wortmannin (Sigma) were added at final concentrations of 2.5, 100, and 1 µM, respectively. Reactions were stopped by adding 10 µl of 5 × Laemmli buffer containing 0.5 × DTT and boiled for 5 min. Proteins were separated by 10% SDS-PAGE, stained, and destained. Phosphorylated proteins were visualized by autoradiography. In some cases, protein bands were excised, and counted on a scintillation counter (MINAXI Tri-Carb 4000, Packard Instrument Co.).

**Phosphorylation of IRS-1 by Recombinant ERK2—**IRS-1 GST fusion proteins were phosphorylated by a linked in vitro recombinant MAP kinase assay (45). The constitutively active form of the MEK protein (kindly provided by Dr. James Posada, University of Vermont) was used in conjunction with either a wild type (ERK2) or a kinase-inactive form of ERK2 (ERK2-kinase-dead) (kindly provided by Dr. James Posada) in kinase buffer (20 mM HEPES, pH 7.5, 10 mM MgCl₂, 1 mM EGTA, and 10 µM [γ-32P]ATP) at 30 °C for 15 min. The reactions were terminated by addition of Laemmli sample buffer and proteins were separated by 10% SDS-PAGE. The phosphorylated substrates were visualized by autoradiography (45).

**Tryptic Peptide Maps and Amino Acid Analysis—**Samples for tryptic peptide maps were phosphorylated in vitro as described for the in vitro kinase assay with the exception that 25 µCi of [γ-32P]ATP and a 2-h incubation time were used. After SDS-PAGE separation, brief staining and destaining, and autoradiography, phosphorylated proteins in the gel were excised, and incubated at 37 °C in 20% methanol for 4 h. Phosphoproteins were extracted by homogenizing the gel fragments in 0.5% SDS and 1 M acetic acid (pH 7.6) and 50 µl of 2 % β-mercaptoethanol, followed by incubating at 100 °C for 5 min and 37 °C for 1.5 h. After centrifugation, phosphoproteins in the supernatants were precipitated by 15% trichloroacetic acid at 4 °C for 1 h, washed with 0.4 ml ethanol/ether (1:1, v/v) twice and air dried (46).

For tryptic peptide maps, trichloroacetic acid precipitants were dissolved in 100 µl of 50 mM ammonium bicarbonate (pH 7.6) containing 0.3 mg/ml t-1-losylamido-2-phenylamino chloromethyl ketone trypsin (Worthington Biochemical Corp.), incubated at 37 °C overnight, dried in a speedvac, and redissolved in Tricine sample buffer (Bio-Rad). Phosphotryptic peptides were resolved by 16.5% Tricine-PAGE and detected by autoradiography (47).

For phosphaomino acid analysis, the trichloroacetic acid precipitates were dissolved in 100 µl of 6 N HCl, hydrolyzed at 110 °C for 1 h, and dried in the speedvac. Residues were dissolved in 10 µl of 0.5 µg/ml standards (phosphotyrosine, phosphoserine, and phosphothreonine mixture). Samples (2 µl) were spotted on a precontd TLC cellulose plate (EM Science) and separated in running buffer (acetic acid:pyridine:water, 10:1:189) by electrophoresis at 1300 V for 40 min on a Hunter thin layer peptide mapping system (HPLC-7000, C.D.S. Scientific). Phospho-L-tyrosine and phospho-threonine standards were visualized by ninhydrin (Sigma) staining (0.25% in acetonitrile), and radioactive amino acid was visualized by autoradiography (49).

**Immunoprecipitation and Immunoblot Analysis—**For whole lysate immunoblot analysis, cells were lysed directly into Laemmli sample buffer containing 0.1 % DTT, sonicated and boiled for 5 min. For MAP kinase immunoblotting, partially purified cell lysates or both wild type
recombinant MAP kinase (ERK2) and kinase-deficient enzyme (ERK2*) were boiled in Lammli sample buffer for 5 min. For immunoprecipitation, cells were lysed in homogenization buffer (20 mM Tris-HCl (pH 7.5), 137 mM NaCl, 100 mM NaF, 1 mM MgCl2, 1 mM CaCl2, 200 μM sodium orthovanadate, 0.4 mM phenylmethylsulfonyl fluoride, 50 μg/ml aprotenin, 50 μg/ml leupeptin, 10% glycerol, and 1% Nonidet P-40 (Calbiochem)) and centrifuged at 13,000 rpm for 15 min (40). The supernatants were incubated with anti-IRS-1 antibody (αIRS-1) or anti-β subunit of the insulin receptor antibody (αIR) (C-19, Santa Cruz), and the immunocomplexes were washed three times with homogenization buffer, and denatured in Laemmli buffer containing 0.1 mM DTT. Proteins in cell lysates or immunoprecipitates were separated by 6 or 10% SDS-PAGE, and transferred to a nitrocellulose membrane. The membrane was 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 either anti-phosphotyrosine antibody (αPY20) or αIR. 32D/IR/IRS-1 and CHO/IR/IRS-1 cells were labeled with [32P]orthophosphate for 4 h in the presence or absence of 100 mM insulin and stimulated with 100 mM insulin for 2 min before being lysed. IRS-1 was immunoprecipitated with αIRS-1 and separated by 7.5% SDS-PAGE. Phosphorylated IRS-1 was visualized by autoradiography.

**RESULTS**

**Chronic Insulin Treatment Decreases Insulin-induced Tyrosine Phosphorylation and Increases Serine/Threonine Phosphorylation of IRS Proteins**—32D cells overexpressing the insulin receptor and IRS-1 or IRS-2 (32D/IR/IRS-1 and 32D/IR/IRS-2) were exposed to control medium or medium containing 100 nM of insulin for 4 or 12 h and then acutely treated with insulin for 2 min. Acute insulin exposure increased tyrosine phosphorylation of both IRS-1 and IRS-2 in the cells exposed to control medium (Fig. 1A, upper panel, lanes a versus b and g versus h), but failed to induce tyrosine phosphorylation of IRS proteins after exposure to 4 or 12 h of insulin (Fig. 1A, upper panel, lanes c–f and i–l). IRS-1 and IRS-2 protein levels were assessed by αIRS-1 or αIRS-2 immunoblotting. There was no significant change of either with the insulin treatment (Fig. 1A, lower panel). The same results were obtained when CHO/IR/IRS-1 cells were exposed to 4 h of insulin although IRS-1 protein level also appeared to slightly decrease (Fig. 1B).

To see whether the reduced tyrosine phosphorylation of IRS proteins is mediated at the level of the insulin receptor, anti-IRS-1, and IRS-2 (32D/IR/IRS-1) cells were exposed to control medium or medium containing 100 nM of insulin for 4 or 12 h and then acutely treated with insulin for 2 min. Acute insulin exposure increased tyrosine phosphorylation of both IRS-1 and IRS-2 in the cells exposed to control medium (Fig. 1A, upper panel, lanes a versus b and g versus h), but failed to induce tyrosine phosphorylation of IRS proteins after exposure to 4 or 12 h of insulin (Fig. 1A, upper panel, lanes c–f and i–l). IRS-1 and IRS-2 protein levels were assessed by αIRS-1 or αIRS-2 immunoblotting. There was no significant change of either with the insulin treatment (Fig. 1A, lower panel). The same results were obtained when CHO/IR/IRS-1 cells were exposed to 4 h of insulin although IRS-1 protein level also appeared to slightly decrease (Fig. 1B).

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Elevated Serine Kinase Activity in Chronic Insulin-treated Cells and in Liver and Muscle of JCR:LA-cp Rats—To determine serine/threonine kinase activity in chronic insulin-treated cells, lysates prepared from CHO/IR/IRS-1 cells were fractionated by (NH₄)₂SO₄ precipitation into 30, 50, and 90% fractions, which were used as sources of kinase to phosphorylate GST-IRS-1 fragments (the N-terminal region of 2–516 amino acids designated IRS-1N, the middle region of 526–859 amino acids designated IRS-1M, and the C-terminal region of 900–1235 amino acids designated IRS-1C (Fig. 2A)) in an in vitro kinase assay. GST-IRS-1N, GST-IRS-1M, and GST-IRS-1C were mainly phosphorylated by the 50% (NH₄)₂SO₄ fractions and very weakly by the 30% and 90% fractions (Fig. 2B). There was no significant difference in the phosphorylation of GST-IRS-1N and GST-IRS-1C by any of the three fractions when the insulin-treated and untreated cells were compared (Fig. 2B, left and right panels). In contrast, insulin treatment (4 and 12 h) significantly enhanced kinase activity in the 50% fraction for the GST-IRS-1M fragment (Fig. 2B, middle panel, lanes e and f versus d). When 3T3-L1 adipocytes, a more physiological relevant cell line, were examined for the effect of chronic insulin treatment, the same result was obtained (Fig. 3A).

Phosphorylation of GST-IRS-1M in vitro by the 50% (NH₄)₂SO₄ fraction was tested in the presence of a protein kinase C inhibitor, protein kinase A inhibitor, and wortmannin, a potent inhibitor for PI 3-kinase. None of the inhibitors altered the chronic insulin-induced serine kinase activity (Fig. 2C).

We next examined kinase activity in lysates prepared from the liver and muscle of JCR:LA-cp lean and obese rats (6 months of age). Lysates were precipitated with 50% saturation of (NH₄)₂SO₄, and kinase activity was measured by the phosphorylation of GST-IRS-1 fragments in our in vitro kinase assay. Consistent with the results from CHO/IR/IRS-1 cells, increased kinase activity of GST-IRS-1M was detected both in the liver and muscle of obese rats, when compared with that of lean animals (Fig. 3B, lanes f versus e and h versus g). In contrast, kinase activity was not different in the lean and obese rats for the GST-IRS-1N and GST-IRS-1C fragments (Fig. 3B, lanes a–d and i–l). This enhanced serine kinase activity is not due to a different amount of proteins applied to our in vitro kinase assay, because 1) the same amounts of total proteins from obese and lean tissues were applied in the assay, 2) Coomassie Blue staining of SDS-PAGE actually showed the same levels of proteins in both obese and lean samples (data not shown), and 3) phosphorylation of GST-IRS-1N or GST-IRS-1C with the lysates preparation from obese and lean tissues showed no significant difference (Fig. 3B, lanes a–d and i–l). One note of caution: the results with liver were reproducible on each determination (n = 6) as opposed to muscle, which at times failed to show observable increased kinase activity. We felt the latter reflects difficulty in preparing and working with tissue extracts from skeletal muscle.

The nature of phosphoamino acids was determined by phosphoamino acid analysis for the GST-IRS-1M phosphorylated by the 50% (NH₄)₂SO₄ fraction prepared from chronic insulin-treated cells and JCR:LA-cp rats. Phosphoserine was the phosphoamino acid in all samples as opposed to virtually no phosphothreonine or phosphotyrosine (Fig. 3C). Phosphoserine content was increased in GST-IRS-1M phosphorylated by chronic insulin-treated cells or tissues of obese rat (Fig. 3C, lanes b, d, f and h versus lanes a, c, e and g). Thus, the enhanced serine kinase activity that phosphorylate the 526–859 amino acid region of IRS-1 was correlated with insulin-resistant cells and animal models.

The in Vitro Phosphorylation of IRS-1 in the Insulin-resis-
enhanced serine kinase activity in liver of obese rat only phosphorylated GST-IRS-1M and GST-C-IRS-1M, and barely phosphorylated GST-N-IRS-1M (Fig. 4B, lanes 17, 18, 27, and 28). Note that the protein level of ERK2 used in the in vitro kinase assay was very similar between CHO/IR/IRS-1 cells and recombinant enzymes, but lower in rat liver and muscle as measured by Western blotting analysis (Fig. 4C). Together, these results clearly show that a serine kinase other than a MAP kinase is activated in the chronic insulin-treated cells and insulin-resistant animals to phosphorylate GST-C-IRS-1M.

Furthermore, if the elevated serine kinase activities in the insulin-resistant state is not MAP kinase, presumably it phosphorylates IRS-1 at different serine sites. To confirm this, we performed phosphopeptide map analysis on phosphorylated GST-IRS-1 fusion proteins. Three major phosphopeptides were identified in phosphorylated GST-IRS-1M, designated P1, P2, and P3, which displayed increased phosphorylation after chronic insulin treatment of CHO/IR/IRS-1 cells with P2 being predominant relative to P1 and P3 (Fig. 4D, lanes c and d). Recombinant ERK2 phosphorylated P1 and P2, but not P3 (Fig. 4D, lane i). Consistent with this data, pretreatment of cells with PD98059 (MEK inhibitor) decreased phosphorylation of P1 and P2 without affecting P3 phosphorylation (Fig. 4D, lanes g and h versus e and f), suggesting that P1/P2 are MAP kinase phosphorylation sites and P3 is likely to be phosphorylated by another serine kinase. Importantly, P3 was observed in all the samples tested, and was predominant in liver of the obese rat and 3T3-L1 adipocytes, but relatively low in muscle of the obese rat relative to P1/P2 (Fig. 4D, lanes a–b and j–m). Interestingly, P1/P2 were not phosphorylated by the enhanced serine kinase from liver of obese rat (Fig. 4D, lanes j and k). Phosphopeptide maps were also analyzed for the GST-N-IRS-1M and GST-C-IRS-1M phosphorylated by the enhanced serine kinase activity from JCR:LA-cp rats and recombinant

**FIG. 3.** Phosphorylation of IRS-1 fragments by lysates from 3T3-L1 adipocytes, liver and muscle of JCR:LA-cp rats and phosphoamino acid analysis. Lysates were prepared from 3T3-L1 adipocytes (A) and liver and muscle isolated from lean and obese rats (B) as described under “Experimental Procedures,” precipitated at the 50% saturation of (NH₄)₂SO₄, and re-suspended in lysate buffer. GST-IRS-1 fragments were phosphorylated with the (NH₄)₂SO₄ precipitates by the in vitro kinase assay. Phosphorylated proteins were separated by 10% SDS-PAGE and visualized by autoradiography. The result is a representative of at least three experiments. C, phosphorylated GST-IRS-1M was extracted from gel, and hydrolyzed by 6 N HCl at 110 °C for 1 h. Phosphoamino acids, together with standards, were separated on a TLC plate and visualized by Ninhydrin staining, and radioactive phosphoamino acids were detected by autoradiography. Phosphoamino acid standards are indicated. The result is a representative of at least two experiments.
ERK2. As expected, P1/P2 in GST-N-IRS-1 M were strongly phosphorylated by recombinant ERK2 and weakly by the serine kinase from muscle of obese rat, whereas the phosphorylation of P1/P2 was undetectable in liver of obese rat (Fig. 4D, lanes n–r). In contrast, GST-C-IRS-1 M contained P3, which is not phosphorylated by recombinant ERK2 at all, but strongly phosphorylated by the liver of obese rat, and weakly by the muscle of obese rat (Fig. 4D, lanes s–x). Taken together, these results suggest that phosphorylation of P1/P2 reflects the MAP kinase activity that is not active at least in the liver of JCR:LA-cp obese rats, whereas P3 is the common phosphopeptide in our insulin-resistant cell and animal models and is phosphorylated by a yet-to-be-identified serine kinase.

DISCUSSION

Serine/threonine phosphorylation of IRS-1 has been implicated in attenuation of insulin action (16, 19–26). To identify serine/threonine kinase activity in insulin-resistant models, we have developed an in vitro kinase assay that is based on using GST-IRS-1 fragments as substrates and partially purified cell extracts as the source of kinase. This avoided the high background encountered with in vivo phosphorylation studies (40).
IRS-1, Insulin Receptor, and Insulin Resistance

and enabled us to measure the changes of serine kinase activity in tissues originating from insulin-resistant animals. By combining the in vitro kinase assay with phosphoprotein peptide mapping analysis, we have identified a novel serine kinase activity designated as P3 serine kinase because it phosphorylates the P3 peptide of IRS-1. This serine kinase activity was elevated in chronic insulin-treated CHO/IR/IRS-1 cells and 3T3-L1 adipocytes, and liver and muscle tissues from JCR:LA-cp obese rats, strongly suggesting that it is a feature of insulin resistance in general. Finally, we provided strong evidence that the P3 serine kinase is neither a MAP kinase nor a PI 3-kinase.

Chronic insulin treatment (mimicking hyperinsulinemia) induces insulin resistance in 3T3-L1 adipocytes, normal rats and healthy human subjects (22, 51–53). In order to investigate serine phosphorylation of IRS-1 in insulin resistance, we first measured tyrosine phosphorylation of IRS-1 and serine kinase activity in culture cells that were chronically treated with insulin. Phosphotyrosine content in IRS proteins was drastically reduced in 32D/IR/IRS-1, 32D/IR/IRS-2, and CHO/IR/IRS-1 cells. Furthermore, IRS-1 and IRS-2 failed to respond to an acute insulin stimulation in terms of tyrosyl phosphorylation. This insensitivity was not due to a defect in the insulin receptor, because receptor autophosphorylation and the receptor protein level were not altered by the chronic insulin treatment. IRS protein levels were unaffected in 32D cells, suggesting that changes in the IRS protein levels does not account for the insensitivity. In contrast, overall phosphate content in IRS-1 was increased by the chronic insulin treatment, suggesting the increased serine and/or threonine phosphorylation of IRS-1. Consistent with this finding, serine kinase activity was elevated in chronically insulin-treated CHO/IR/IRS-1 cells and 3T3-L1 adipocytes. Of greatest potential significance, high serine kinase activity was also detected in liver and muscle tissues from insulin-resistant JCR:LA-cp rats. Viewed together, our results show a consistent finding of IRS-1 serine phosphorylation in both in vitro and in vivo insulin-resistant states.

We also investigated possible candidates for the serine kinase. Activation of protein kinase C induces serine phosphorylation of IRS-1 and attenuates insulin action (48). This stems from the activation of MAP kinase, which phosphorylates IRS-1 at Ser\(^{612}\) in vitro and in vivo (35, 36). Our results showed that the enhanced serine kinase activity in the insulin-resistant cells and tissues specifically phosphorylated GST-IRS-1m but not GST-IRS-1\(^{1-54}\) and GST-IRS-1\(^{1-6}\). GST-IRS-1\(^{1-6}\) contains two consensus sequences for MAP kinase (P\(^{x\text{SP}}\), Ser\(^{612}\) and Ser\(^{632}\). By phosphopeptide mapping, three major phosphopeptides (P1, P2, and P3) were identified in GST-IRS-1\(^{1-6}\) that could be phosphorylated by the serine kinase activity in cells and tissues of insulin-resistant models. P1 and P2, but not P3, were phosphorylated by recombinant MAP kinase (ERK2), and therefore, they most likely represent MAP kinase activity. However, P1 and P2 phosphorylation did not perfectly correlate with the serine phosphorylation in all of the tested models, particularly in the liver of insulin-resistant animal, which argues against MAP kinase being the serine kinase that phosphorylates IRS-1 in insulin-resistant states.

The serine kinase that phosphorylates P3 is unknown and was tentatively designated P3 serine kinase. P3 serine kinase was significantly enhanced in all of the tested insulin-resistant samples and in fact is the most consistent observation across all of our systems. The current study provides definitive evidence against MAP kinase being operative in the phosphorylation of P3 because 1) the P3 phosphopeptide was identified in amino acids 721–859 region of IRS-1 (GST-C-IRS-1\(^{1-6}\)), which contains no phosphorylation sites for MAP kinase, 2) recombinant MAP kinase failed to phosphorylate GST-C-IRS-1\(^{1-6}\) and P3 peptide, and 3) pretreatment of cells with MEK inhibitor did not decrease P3 serine kinase activity in chronic insulin-treated CHO/IR/IRS-1 cells. We do not believe that it is PI 3-kinase, either, because wortmannin had no effect on the serine kinase activity. Moreover, activation of MAP kinase (22, 54) and PI 3-kinase (22, 55–57) has been reported to be impaired in insulin-resistant cultured cells, insulin-resistant mice, and diabetes. Thus, the identity of P3 serine kinase remains unknown.

In summary, we found a novel elevated serine kinase activity that phosphorylates IRS-1 in lysates from insulin-resistant animals and cell models. The serine phosphorylation site was located in the 721–859 region of IRS-1. We speculate that serine phosphorylation of this region of IRS-1 impairs its functional role in insulin action perhaps through altering tyrosyl phosphorylation of IRS-1. Serine phosphorylation of IRS-1 seems to be important for the binding of IRS-1 to 14-3-3 protein (58–60). Interestingly, the amino acids 516–865 region of IRS-1, which has been found to interact with 14-3-3, is similar to the corresponding region of IRS-1\(^{1-6}\) (amino acids 526–859). 14-3-3 proteins have been found to associate with a number of signaling proteins, including Raf, Bcr-Abl, poloma virus middle T antigen, PI 3-kinase, the proto-oncogene product Cbl, and cdc25 phosphatase, implicating an important role in mitogenesis or cellular transformation (60). Thus, the serine phosphorylation of IRS-1 in insulin-resistant states may lead to altering the binding of 14-3-3 proteins. We were currently investigating this possibility. Purification of P3 serine kinase and determination of the phosphorylation sites within the 721–859 region responsible for the attenuation of insulin signaling should greatly facilitate the investigation of the role of serine phosphorylation of IRS proteins in insulin action.

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