Protein Kinase C Modulation of Insulin Receptor Substrate-1 Tyrosine Phosphorylation Requires Serine 612[†]

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ABSTRACT: Activation of the endogenous protein kinase Cs in human kidney fibroblast (293) cells was found in the present study to inhibit the subsequent ability of insulin to stimulate the tyrosine phosphorylation of an expressed insulin receptor substrate-1. This inhibition was also observed in an *in vitro* phosphorylation reaction if the insulin receptor and its substrate were both isolated from cells in which the protein kinase C had been activated. To test whether serine phosphorylation of the insulin receptor substrate-1 was contributing to this process, serine 612 of this molecule was changed to an alanine. The insulin-stimulated tyrosine phosphorylation and the associated phosphatidylinositol 3-kinase activity of the expressed mutant were found to be comparable to those of the expressed wild-type substrate. However, unlike the wild-type protein, activation of protein kinase C did not inhibit the insulin-stimulated tyrosine phosphorylation of the S612A mutant nor its subsequent association with phosphatidylinositol 3-kinase. Tryptic peptide mapping of *in vivo* labeled IRS-1 and the S612A mutant revealed that PMA stimulates the phosphorylation of a peptide from wild-type IRS-1 that is absent from the tryptic peptide maps of the S612A mutant. Moreover, a synthetic peptide containing this phosphoserine and its nearby tyrosine was found to be phosphorylated by the insulin receptor to a much lower extent than the same peptide without the phosphoserine. Activation of protein kinase C was found to stimulate by 10-fold the ability of a cytosolic kinase to phosphorylate this synthetic peptide as well as the intact insulin receptor substrate-1. Finally, cytosolic extracts from the livers of *ob/ob* mice showed an 8-fold increase in a kinase activity capable of phosphorylating this synthetic peptide, compared to extracts of livers from lean litter mates. These results indicate that activation of protein kinase C stimulates a kinase which can phosphorylate insulin receptor substrate-1 at serine 612, resulting in an inhibition of insulin signaling in the cell, posing a potential mechanism for insulin resistance in some models of obesity.

Insulin receptor substrate-1 (IRS-1)[1] is a major endogenous substrate of the insulin receptor tyrosine kinase (Lee & Pilch, 1994; White & Kahn, 1994). Tyrosine-phosphorylated IRS-1 appears to serve as a docking protein for a number of known Src homology (SH) 2 domain-containing proteins such as Grb2, Syp, Nck, and two isoforms of the regulatory subunit of PI 3-kinase (p85α and p85β) (Lee & Pilch, 1994; White & Kahn, 1994). Binding of tyrosine-phosphorylated IRS-1 to the PI 3-kinase leads to a 3−5-fold stimulation of its enzymatic activity, presumably causing the insulin-stimulated increase of PI 3,4-bisphosphate and 3,4,5-trisphosphate in the cell (Rordorf-Nikolic et al., 1995; Ruderman et al., 1990). Through presently unknown downstream signaling components, activation of PI 3-kinase appears to lead to several of the final effects of insulin such as stimulation of glucose uptake and activation of various serine/threonine kinases such as the 70 kDa S6 kinase (Cheatham et al., 1994; Chung et al., 1994). Although IRS-1 contains at least 20 potential tyrosine phosphorylation sites (Sun et al., 1991), 1 (tyrosine 608) appears to be the principal site of interaction with the SH2 domain of PI 3-kinase while 3 others (tyrosines 460, 939, 987) play additional roles (Rocchi et al., 1995).

IRS-1 also undergoes extensive serine/threonine phosphorylation; the migration of IRS-1 on SDS−PAGE at an electrophoretic mobility corresponding to 165−185 kDa, compared to its predicted molecular mass of 131 kDa, is consistent with the high level of serine/threonine phosphorylation of IRS-1 observed in the unstimulated state (Sun et al., 1991). In addition, insulin stimulation of cells as well as activation of protein kinase C results in an increase in the serine/threonine phosphorylation of IRS-1 (Sun et al., 1991; Chin et al., 1994). More recently, excessive serine/threonine phosphorylation of IRS-1 has been proposed to be one mechanism whereby tumor necrosis factor induces resistance to insulin (Hotamisligil et al., 1996; Kanety et al., 1995).

Since insulin resistance appears to be a contributing factor in the development of non-insulin-dependent diabetes mellitus, extensive studies have attempted to define the detailed biochemical mechanisms for this resistance (Olefsky & Nolan, 1995). To this end, several cellular systems have been examined, including treatment of cells with high concentrations of insulin for long periods of time, activation of protein kinase C, or treating cells with high glucose or inhibitors of serine/threonine phosphatases such as okadaic acid (Pillay et al., 1996; Takayama et al., 1988; Tanti et al., 1994; Treadway et al., 1989). In the latter system, increased

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[1] Abbreviations: IRS, insulin receptor substrate; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; IR, insulin receptor; PI, phosphatidylinositol.

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serine/threonine phosphorylation of IRS-1 has been observed to contribute to the decreased ability of insulin to stimulate the tyrosine phosphorylation of IRS-1 and its subsequent association with the PI 3-kinase. However, an attempt to prove this hypothesis by mutation of potential serine phosphorylation sites in the IRS-1 molecule was unsuccessful (Mothe & Van Obberghen, 1996), possibly due to the large number of serine/threonine kinases activated in this system and hence causing a complex pattern of serine/threonine phosphorylation of IRS-1.

In the present studies, we have examined the role of serine/threonine phosphorylation of IRS-1 in the insulin resistance observed in cells after activation of protein kinase C (PKC). As noted above, activation of PKC has been found to inhibit insulin-stimulated signaling in a variety of systems, and this inhibition is potentiated by the overexpression of particular PKC isoenzymes (Chin et al., 1993). The activation of PKC has been shown to cause an increase in the serine/threonine phosphorylation of the IR at a number of residues although the important regulatory site(s) has (have) not yet been identified (Lewis et al., 1994; Liu & Roth, 1994). Moreover, activation of PKC has been observed in tissues of hyperglycemic animals and humans (Considine et al., 1995; Donnelly et al., 1994; Xia et al., 1994). Finally it has been shown that activation of PKC stimulates the serine/threonine phosphorylation of IRS-1 (Chin et al., 1994). Here we show that the activation of the endogenous PKC in human kidney fibroblast (293) cells is sufficient to inhibit the insulin-stimulated tyrosine phosphorylation of a stably expressed epitope-tagged IRS-1 (IRSmyc) both in an intact cell and *in vitro*, indicating that this inhibition is due to a stable modification of the IRS-1 protein. We also show that both the *in vivo* and *in vitro* PKC inhibition of insulin-stimulated tyrosine phosphorylation of IRS-1 is lost in a mutant IRS-1 whose serine residue 612 has been changed to alanine, and that PMA treatment of cells expressing IRS-1 results in phosphorylation of a tryptic peptide that is not phosphorylated in the S612A mutant, consistent with the hypothesis that the PKC-mediated insulin resistance in this system is caused by increased serine/threonine phosphorylation of IRS-1. Moreover, we show that a synthetic peptide in which the serine 612 is replaced with a phosphoserine is phosphorylated by the isolated IR *in vitro* to a much lesser degree than a comparable peptide without this phosphate group. Finally, PMA treatment causes a 10-fold increase in enzymatic activity of a cytosolic kinase activity that phosphorylates both full-length IRS-1 and the IRS-1 peptide containing S612. A similar increase in kinase activity is found in the livers of *ob/ob* mice when compared to their lean littermates. These results demonstrate that protein kinase C modulation of insulin signaling is partly mediated via an increase in serine phosphorylation of IRS-1.

EXPERIMENTAL PROCEDURES

Materials

Monoclonal anti-myc 9E10 antibody for immunoprecipitation was purchased from Berkeley Antibody Co. Monoclonal anti-IRS-1 hybridoma supernatant was a gift from Dr. Kazuyoshi Yonezawa. Polyclonal anti-IRS-1 (C-terminus) antibody for Western blot analysis was purchased from Upstate Biotechnology Inc. Anti-phosphotyrosine antibody (RC20) coupled to horseradish peroxidase was obtained from Transduction Laboratories. Protein G−agarose was purchased from Pharmacia. [γ-^{32}P]ATP was purchased from Amersham Co. Purified PI was obtained from Avanti Biochemicals. Phorbol 12-myristate 13-acetate (PMA) was purchased from Calbiochemical Co. The double-stranded mutagenesis kit was obtained from Clontech. Oligonucleotides were obtained from Anagen Biotechnologies, Genemed Biotechnologies, or OPERON Biotechnologies. Plasmid preparation and DNA purification kits were purchased from QIAGEN. Enzymes for molecular biology were obtained from New England Biolabs and Bethesda Research Laboratories. Pfu I polymerase for PCR was obtained from Stratagene. SDS−PAGE reagents and lipofectin reagent for stable transfections were purchased from GIBCO. IRS-1 peptides were synthesized by the Beckman PAN facility. The *ob/ob* and lean mice were supplied by Receptron. All other reagents were purchased from Sigma.

Methods

Epitope Tagging and Mutagenesis of IRS-1. The rat IRS-1 cDNA (Sun et al., 1991) in pBluescript IIKS was a gift from Dr. M. F. White (Joslin Diabetes Center, Boston, MA), and was subcloned into pBluescript IISK such that the *Kpn*I site in the polylinker was located 5′ to the IRS-1 gene. The IRS-1 coding region was excised from pBSK/IRS by digestion with *Sac*II and *Hpa*I and ligated into the *Sac*II and *Hpa*I sites in the pCLDN expression vector (a gift of Drs. John Trill and Dave Pfarr, Smith Kline Beecham). To make the expressed IRS-1 distinguishable from the endogenous IRS-1, the myc epitope was engineered onto the N-terminus of IRS-1 by PCR using a forward primer [GGGGTCGACATGGAGGAACAGAAACTTATCTCGGAAGAAGATCTGCTGCGTAAGCGTGCGAGCCCTCCGG] comprised of 13 perfectly matched base pairs to nucleotides 592−605 of rat IRS-1 preceded by 39 nucleotides encoding the myc epitope, an ATG in a perfect Kozak consensus sequence, and a *Sal*I site for insertion into the expression vector. The reverse primer is complementary to nucleotides 934−962 of rat IRS-1. A 420 base pair fragment was obtained by PCR, purified, cut with the restriction enzymes *Sal*I and *Xho*I, and ligated into pBSK/IRS-1 digested with *Xho*I to yield pBS/IRSmyc. pBS/IRSmyc was digested with *Kpn*I; a 320 base pair fragment was purified from 1% agarose and ligated into pCLDN/IRS-1 digested with *Kpn*I to yield pCLDN/IRSmyc. To obtain point mutants of IRS-1, a *Bgl*II to *Hin*dIII fragment from pCLDN/IRS-1 was ligated into puc18. Double-stranded site-directed mutagenesis was performed using the Clontech double-strand DNA strategy, based on the Deng and Nickloloff method (Deng & Nickoloff, 1992). The mutagenic oligonucleotide [CACTGGAGCCACCCCGGGAGCCATGGG] was used to change a serine at codon 612 to an alanine. The mutated region was excised with *Age*I and *Bam*HI digestion and subcloned into pCLDN/IRSmyc, digested with *Age*I and *Bam*HI to yield pCLDN/IRSmyc-S612A. The reconstructed plasmid was confirmed by restriction mapping and sequencing from nt 2120 through 2510.

Cell Culture and Transfections. Wild-type and mutant IRS-1 were either transiently or stably expressed in 293T (a 293 line which constitutively expresses T-antigen) or 293 cells, respectively. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS). Transient transfections were performed in

293T cells using the calcium phosphate precipitation method of Chen and Okayama (1987), with modifications. Fourteen to seventeen hours after transfection, cells were incubated in DMEM containing 10% FCS. Stable transfections were performed with 293 cells using Lipofectin Reagent, followed by selection with 1 mg/mL geneticin. Single colonies were isolated and screened for expression of IRSmyc and IRSmyc S612A by Western blotting with antibodies to myc. Four clones, two expressing wild type and two expressing mutant IRSmyc, were identified and maintained in DMEM/10% FCS, supplemented with 0.5 mg/mL geneticin.

In Situ Tyrosine Phosphorylation of IRS-1. Ten centimeter dishes of 293 cells stably expressing either IRSmyc or IRSmycS612A were serum-starved for 16 h and then treated with or without PMA for 20 min followed by 7 min in the presence or absence of 1 μM insulin. Cells were washed 1 time in 50 mM Hepes (pH 7.6)/150 mM NaCl and lysed on ice for 20 min in lysis buffer A (50 mM Hepes, pH 7.6, 150 mM NaCl, 1% Triton X-100, 2 mM vanadate, 100 mM NaF, 2.5 mM sodium pyrophosphate, 10 mM EDTA, 0.25 mM PMSF, and 10 μg/mL aprotinin), and adsorbed with anti-myc antibodies bound to protein G−agarose to isolate IRS-1. After 3 h at 4 °C, the agarose beads were washed 2 times in buffer (50 mM Hepes, pH 7.6, 150 mM NaCl, 0.1% BSA, and 0.1% Triton X-100) and 1 time in phosphate-buffered saline, and the bound proteins were eluted by boiling in gel loading buffer (40% glycerol, 1% SDS, 50 mM Tris, pH 6.8, and 1 M DTT). After separation of the samples by SDS−PAGE and transfer to nitrocellulose, the membrane was probed with an anti-phosphotyrosine antibody coupled to horseradish peroxidase, and analyzed by enhanced chemiluminescence (ECL). Membranes were stripped of anti-phosphotyrosine antibodies by incubation in 2% phenyl phosphate in Tris/NaCl for 3 h, washed 2 times, and reprobed with a polyclonal antibody to either IRS-1 or myc, followed by visualization by secondary antibodies coupled to alkaline phosphatase and development with chromagenic substrates. Quantitation of the bands was performed by scanning, and the amount of tyrosine phosphorylated IRS-1 was normalized to the total amounts of IRS-1 present in each lane and fold insulin stimulation was calculated as the level of tyrosine phosphorylation in the immunoprecipitates from insulin-treated cells versus untreated cells.

PI 3-Kinase Activity. 293 cells stably expressing either wild-type or mutant IRS-1 were treated as described above and then lysed (20 mM Tris, pH 8.0, 137 mM NaCl, 1% Nonidet-P40, 10% glycerol, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 200 μM vanadate, 1 mM PMSF, and 10 μg/mL aprotinin). The lysates were immunoprecipitated with either anti-myc antibodies, normal mouse Ig, or monoclonal anti-p85 bound to protein G−agarose, the beads were washed, incubated with 10 μg of PI and 0.5 μCi of [γ-^{32}P]ATP for 5 min at room temperature, the reaction was terminated, and the phosphorylated PI was analyzed by thin-layer chromatography as described previously (Chin et al., 1994). Bands were excised from the plates and counted.

In Vitro Phosphorylation of IRS-1. Ten centimeter plates of confluent 293T cells transiently transfected with IRSmyc or stably transfected 293 cells were serum-starved for 2 h and treated either with or without PMA for 20 min. Cells were lysed in lysis buffer (as described above but without EDTA) for 20 min, and IRS-1 either was captured on microtiter plates coated with anti-myc antibody as previously described (Boge & Roth, 1995) or was obtained by immunoprecipitation with anti-myc antibodies adsorbed to protein G−agarose. IRS-1 from approximately 2×10^5 cells was added to each well or to each set of beads, respectively. IR was prepared by incubation of the lysates with wheat germ agglutinin coupled to agarose and eluted with 0.3 M *N*-acetylglucosamine as previously described (Boge & Roth, 1995). Either 10 μL of eluted IR, corresponding to IR captured from approximately 4×10^5 cells, or 10 μL of the elution buffer was added to each IRS-1 sample in the presence of 1 μM insulin, 1 mM ATP, 10 mM $MgCl_2$, and 10 mM $MnCl_2$ and incubated at room temperature for 20 min. Beads were washed 3 times in 50 mM Hepes/150 mM NaCl/1% BSA, and IRS-1 was eluted and analyzed as described above. Microtiter plates were washed with the same buffer and incubated with anti-phosphotyrosine antibodies for 1 h. Bound anti-phosphotyrosine antibodies were determined by ELISA as previously described (Boge & Roth, 1995).

In Vitro Peptide Assays. 293 cells were serum-starved for 2 h, and IR was prepared as described for *in vitro* IRS-1 phosphorylation. Eluted IR was activated by incubation with 1 μM insulin for 30 min at 4 °C, followed by incubation with 5 mM $MnCl_2$ and 50 μM ATP for 30 min. IRS-1 peptides (KKHTDDGYMPMSPGVA or KKHTDDGYMP-MpSPGVA) at concentrations ranging from 0 to 1.2 mM were incubated in a final volume of 40 μL with activated IR in a reaction mixture containing 50 mM Hepes, 150 mM NaCl, 5 mM $MnCl_2$, 10 μM ATP, and 0.5 μCi of [γ-^{32}P]-ATP (final concentrations). After 10 min at 25 °C, reactions were stopped by first adding 5 μL of 3% BSA carrier protein rapidly followed by 65 μL of 3% TCA. After 30 min on ice, the mixture was spun for 5 min in a microcentrifuge to remove IR. Peptide-containing supernatants were then analyzed either by addition of 5 μL of 2 M Tris base and equal volumes of 6 M urea and 125 mM Tris (pH 6.8), followed by electrophoresis on 40% polyacrylamide gels and autoradiography, or by spotting onto phosphocellulose paper (P81, Whatman); the paper was washed 3 times in 1% phosphoric acid and counted.

Tryptic Peptide Maps of IRS-1. Ten centimeter plates of 293/IRS and 293/S612A IRS cells were grown to confluence (approximately 2 days). Cells were then serum-starved for 6 h, washed 3× in Krebs−Ringer−phosphate-free medium (118 mM NaCl, 4.75 mM KCl, 1.2 mM $MgCl_2$, 260 nM $CaCl_2$, 25 mM $NaHCO_3$, 50 mM glucose, 20 mM Hepes, and 1% penicillin/streptomycin), and incubated with 500 μCi of [^{32}P]orthophosphate in 3 mL of Krebs−Ringer solution for 4 h. PMA at a final concentration of 2 μM was added for the final 20 min of the labeling. Cells were lysed in lysis buffer A with the addition of 300 nM okadaic acid for 20 min on ice and immunoprecipitated with anti-myc antibodies bound to protein G for 2 h. Immunoprecipitated proteins were washed 2 times in WGBT, 1 time in 100 mM Tris-HCl (pH 7.6)/1 M NaCl, and 1 time in PBS, eluted, and analyzed on 7.5% PAGE followed by transfer to Immobilon-P membranes. Radiolabeled IRS-1 was visualized by autoradiography, and the IRS-1 bands were cut out, counted, blocked with 0.5% polyvinylpyrrodoline 40 000 for 30 min at 37 °C, and washed 5 times with H_2O and twice with 50 mM ammonium bicarbonate in 100 mM acetic acid. Membranes were then incubated with 50 μg of trypsin in 400 μL of 50 mM ammonium bicarbonate for 6 h at 37 °C;

an additional 25 μg of trypsin was added and incubated overnight. Samples were spun for 5 min and supernatants transferred to fresh tubes; approximately 60% of the radiolabel was recovered in the supernatants. Samples were lyophilized, resuspended in 500 μL of H_2O, and lyophilized again. Pellets were resuspended in 25 μL of pH 1.9 buffer and centrifuged for 30 min, and supernatants were transferred to fresh tubes. Samples were spotted onto cellulose plates and electrophoresed at 1 kV in pH 1.9 buffer for 2 h. Plates were dried and subjected to chromatography in the second dimension for 7 h (Boyle et al., 1991). Plates were dried and autoradiographed.

Assay of Cytosolic Kinase Activity. 293 cells were serum-starved 2−18 h followed by 20 min incubations with or without 3 μM PMA. Cells were scraped in buffer A (20 mM Tris, pH 7.6, 220 mM sucrose, 10 mM EDTA, 10 mM $NaPP_i$, 2 mM sodium vanadate, 10 mM NaF, 10 μg/mL aprotinin, 1 mM PMSF, and 250 μM okadaic acid), homogenized 15 strokes, sonicated 3 times for 15 s, and centrifuged at 200 000*g* for 20 min. The supernatants (the cytosol) were removed, and Hepes (60 mM, final concentration) and $MgCl_2$ (10 mM) were added. Lysates from mouse livers were prepared by homogenizing 250 mg of liver in 1 mL of buffer A with a polytron homogenizer 30 s on ice. Samples were sonicated and centrifuged as described for cell lysates. The cytosol (60 μL) was added to reaction tubes containing either immunoprecipitated IRS-1 or varying concentrations of a biotinylated IRS-1 peptide (biotin-T-D-D-G-Y-M-P-M-S-P-G-V-A) or the nonbiotinylated peptide (K-K-H-T-D-D-G-Y-M-P-M-S-P-G-V-A) and 20 mM Tris, pH 7.6, 3.6 mM $MgCl_2$, 1.8 mM $MnCl_2$, 15 μM ATP, and 0.5 μCi of [γ-^{32}P]ATP. After 30 min at 24 °C, reactions were terminated by the addition of either 750 μL of buffer B (buffer A + 1% NP40 and 25 mM ATP) or 100 μL of buffer U (125 mM Tris, pH 6.8, 6 M urea). Biotinylated peptides were captured by incubation with streptavidin−agarose at 4 °C for 2 h, after which beads were washed 3 times in high-salt wash buffer (1 M NaCl, 100 mM Tris, pH 8) and counted. Nonbiotinylated peptides were analyzed by electrophoresis on a 40% acrylamide/urea gel and autoradiography, and the bands were excised and counted. Immunoprecipitated IRS-1 was washed 3 times in high-salt wash buffer and analyzed by electrophoresis on a 7.5% polyacrylamide gel and transferred to nitrocellulose followed by autoradiography. The total amount of IRS-1 protein present in the bands was then measured by immunoblotting with anti-IRS-1 antibodies. The bands were then excised and counted.

RESULTS

PKC Activation Inhibits Insulin Signaling in 293 Cells. Human kidney fibroblasts (293 cells), stably expressing a myc-tagged IRS-1, exhibited a 5-fold inrease in IRS-1 tyrosine phosphorylation with insulin treatment (Figure 1A). An epitope (myc)-tagged IRS-1 was utilized so that the expressed IRS-1 could be distinguished from the endogenous IRS-1. When these cells were pretreated with an activator of PKC, PMA, we observed an inhibition of the subsequent ability of insulin to stimulate the tyrosine phosphorylation of IRS-1 (Figure 1). Thus, the endogenous PKCs present in these cells appeared to be sufficient to mediate an inhibition of IR signaling. To determine whether this inhibition was due to a stable modification of either the IR

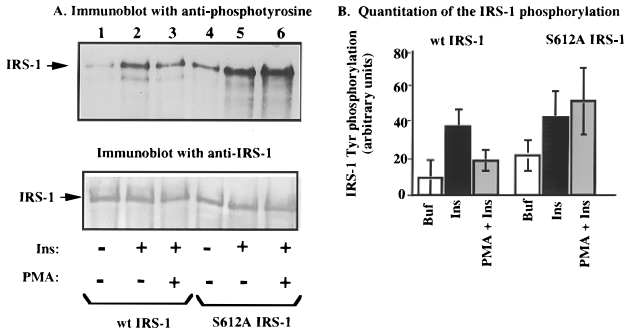

FIGURE 1: Effect of PKC activation on the insulin-induced tyrosine phosphorylation of wild type (wt) IRS-1 and S612A mutant IRS-1 *in situ*. (A) Immunoblot with anti-phosphotyrosine and anti-IRS-1 antibodies of *in situ* phosphorylated IRS-1. Cells stably expressing either the myc-tagged wild-type (wt) IRS-1 (lanes 1−3) or the S612A mutant IRS-1 (lanes 4−6) were pretreated with either PMA or buffer (Buf) as indicated, followed by insulin (Ins), and lysed, and the lysates were immunoprecipitated with anti-myc antibodies. The precipitates were analyzed by SDS−PAGE and immunoblotting with anti-phosphotyrosine antibody (upper panel), followed by stripping and probing with anti-IRS-1 antibody (lower panel). (B) Quantitation of the insulin-stimulated *in situ* tyrosine phosphorylation of IRS-1. The results from four experiments like that shown in (A) were pooled and are plotted as the mean amount of IRS-1 tyrosine phosphorylation ± SEM after normalization for the amount of IRS-1 present in each lane.

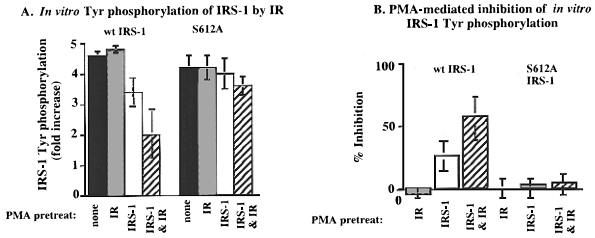

FIGURE 2: Effect of PKC activation on the insulin-induced tyrosine phosphorylation of wild-type (wt) IRS-1 and S612A mutant IRS-1 *in vitro*. (A) Insulin-stimulated *in vitro* tyrosine phosphorylation of IRS-1. Intact 293 cells expressing myc-tagged IRS-1 or S612A mutant IRS-1 were treated with or without PMA for 20 min as indicated and lysed, and the IRS-1 was immunoprecipitated and used in an *in vitro* phosphorylation reaction. The IR was also isolated from cells that were pretreated with or without PMA as indicated. The tyrosine phosphorylation of IRS-1 was analyzed either by SDS−PAGE and immunoblotting or by ELISA. The results from 7 experiments (4 gels and 3 ELISA) were pooled and are plotted as the mean amount of IRS-1 tyrosine phosphorylation ± SEM. after normalization for the amount of IRS-1 present in each reaction. (B) Percent inhibition of the IRS-1 tyrosine phosphorylation. The extent of inhibition by PMA pretreatment was calculated for the data shown in panel A for the wild-type and mutant IRS-1.

or the IRS-1 in these cells, we attempted to reconstitute this inhibition *in vitro*. Receptors from cells treated or not with PMA were isolated and incubated *in vitro* with insulin, ATP, and IRS-1 isolated from cells treated with or without PMA. The extent of IRS-1 tyrosine phosphorylation was assessed either by Western blotting with anti-phosphotyrosine antibodies or by ELISA (Boge & Roth, 1995). In each case, the total amount of IRS-1 was also determined and the results were normalized for the amount of IRS-1 present. IRS-1 prepared from untreated cells was phosphorylated to the same extent by IR prepared either from nontreated cells or from PMA-treated cells (Figure 2A). In contrast, IRS-1 from PMA-treated cells was not phosphorylated as well by IR prepared from PMA-treated cells (Figure 2A). These results indicate that the effect of PKC activation is mediated at the

level of both IRS-1 and IR, and are consistent with the hypothesis that IRS-1 is stably modified in the cells with activated PKC.

Effect of S612A Mutation on PKC-Mediated Inhibition of IRS-1 Tyrosine Phosphorylation. Since the *in vitro* studies described above indicate that the IRS-1 isolated from PMA-treated cells is stably modified, they suggest that IRS-1 could be serine-phosphorylated after PKC activation. Several serine residues in IRS-1 lie adjacent to identified tyrosine phosphorylation sites (for example, serines 612, 632, 662, and 731) (Sun et al., 1991; White & Kahn, 1994) and also are located in the motif -P-X-S/T-P- recognized by serine kinases such as the stress-induced kinases, ceramide-activated kinase, and extracellular regulated kinases (ERK) (Liu et al., 1994; Mukhopadhyay et al., 1992; Trigon & Morange, 1995). Serine 612 in particular is only four amino acids from tyrosine 608, a residue known to be phosphorylated by the IR and play a major role in PI 3-kinase binding (Rocchi et al., 1995). For this reason, we examined the effect of changing serine 612 to an alanine (S612A mutation) on the PKC-mediated inhibition of insulin signaling. Stably transfected 293 cell lines expressing comparable levels of either wild-type IRSmyc or the S612A mutation (293/IRS and 293/S612A, respectively) were established. Cells were treated with or without PMA and insulin, the expressed IRS-1 was specifically immunoprecipitated via the use of an antibody to the epitope tag, and the extent of IRS-1 tyrosine phosphorylation was assessed by immunoblotting with antiphosphotyrosine antibodies. Insulin stimulated the tyrosine phosphorylation of the S612A mutant IRS-1 as well as the wild-type IRS-1 (Figure 1A). The same blots were stripped and reprobed with an antibody to IRS-1 so that the levels of total IRS-1 could be measured (Figure 1A, lower panel). The pooled results from four experiments showed that the maximal levels of insulin-stimulated tyrosine phosphorylation of the wild-type and mutant IRS-1 molecules per total IRS-1 were about the same; however, the basal level of tyrosine phosphorylation of the S612A mutant was greater than the wild-type IRS-1 (Figure 1B). One explanation for this observation would be that the wild-type IRS-1 normally has a certain basal level of phosphorylation of serine 612 which inhibits the basal level of tyrosine phosphorylation of this molecule.

More important for the purposes of the present studies was the finding that while pretreatment with PMA reduces the amount of insulin-stimulated tyrosine phosphorylation of wild-type IRS-1, PMA pretreatment has no effect on the ability of insulin to stimulate the tyrosine phosphorylation of the S612A mutant IRS-1 (Figure 1). This experiment was repeated 4 times with two separate clones for 293 cells expressing either the wild-type IRS-1 or the S612A mutant IRS-1. Results from four experiments were pooled and depicted as the amount of tyrosine-phosphorylated IRS-1 in insulin-treated cells compared to untreated cells. PMA pretreatment resulted in a $45 \pm 9\%$ ($n = 4$) inhibition in the insulin-stimulated tyrosine phosphorylation of wild-type IRS-1 but had no significant effect on insulin-stimulated tyrosine phosphorylation of the S612A mutant IRS-1. The levels of total IRS-1 in each experiment were measured, and the data on IRS-1 tyrosine phosphorylation have been normalized for IRS-1 levels.

A different mutant IRS-1 (in which serine 731 had been changed to alanine) was constructed, and stable cell lines expressing this mutant IRS-1 showed the same PKC-mediated inhibition as the wild-type IRS-1 (data not shown). Serine 731, like serine 612, is only four amino acids away from a tyrosine residue (tyrosine 727) known to be phosphorylated by IR, but tyrosine 727 is not essential for PI 3-kinase binding (Rocchi et al., 1995; White & Kahn, 1994). The inability of the S731A mutation to reverse the PKC-mediated inhibition shows the specificity of this effect.

The ability of IR to tyrosine-phosphorylate *in vitro* the S612A mutant IRS-1 compared to wild-type IRS-1 was investigated using the assay described above. The S612A mutant IRS-1 from untreated cells is phosphorylated to the same extent as wild-type IRS-1 by IR prepared from either untreated or PMA-treated cells (Figure 2). In contrast to the wild-type IRS-1, however, the S612A mutant from PMA-treated cells was still tyrosine-phosphorylated by IR prepared from PMA-treated cells (Figure 2A). When the results of seven *in vitro* studies were quantitated and pooled, wild-type and mutant IRS-1 exhibited a (4.8 ± 1.5)- and a (4.2 ± 1.4)-fold increase in tyrosine phosphorylation, respectively, upon incubation with IR. IR from PMA-treated cells equally phosphorylated the mutant and wild-type IRS-1. However, IR from PMA-treated cells exhibited a $60 \pm 15\%$ ($n = 7$) inhibition in its ability to phosphorylate wild-type IRS-1 from PMA-pretreated cells whereas this same receptor preparation showed no decrease in its ability to phosphorylate the mutant IRS-1 from PMA-treated cells (Figure 2B).

Effect of S612A Mutation on PKC-Mediated Inhibition of IRS-1 Associated PI 3-Kinase Activity. To determine whether the PKC-mediated inhibition of the insulin-stimulated tyrosine phosphorylation of IRS-1 had a functional consequence, we examined the insulin-stimulated association of IRS-1 with the PI 3-kinase. Insulin greatly stimulated the amount of PI 3-kinase activity associated with both the wild-type and S612A mutant IRS-1 (Figure 3A). The amount of PI 3-kinase associated with the S612A mutant IRS-1 from insulin-treated cells was higher than that observed with the wild-type IRS-1 in some experiments (Figure 3), possibly due to the presence of basal levels of phosphorylation at serine 612 in the wild-type receptor but not the mutant. PMA pretreatment inhibited by $50 \pm 7\%$ ($n = 4$) the amount of PI 3-kinase activity found associated with the wild-type IRS-1 in insulin-treated cells (Figure 3). In contrast, PMA pretreatment had no effect on the amount of PI 3-kinase activity found associated with the S612A mutant IRS-1 in insulin-treated cells (Figure 3). This difference was not due to variations in the expression of PI 3-kinase in the cell lines as the total activity present in anti-PI 3-kinase precipitates from the different cells was equivalent (data not shown).

Comparison of the Tryptic Peptide Maps of the in Vivo Labeled Wild-Type and S612A Mutant IRS. To determine whether or not S612 was phosphorylated by PMA treatment *in vivo*, we compared tryptic peptide maps of wild-type and S612A mutant IRS-1. 293/IRS or 293/S612A cells were labeled for 4 h with [^{32}P]orthophosphate and then treated with PMA for 20 min. Cells were lysed, and the IRS-1 was immunoprecipitated with anti-myc antibodies and analyzed by SDS−PAGE followed by transfer to an Immobilon membrane. Radiolabeled IRS-1 bands were visualized by autoradiography, excised, and digested with trypsin, after which they were spotted onto TLC plates and anaylzed in the first dimension by electrophoresis in pH 1.9 buffer and

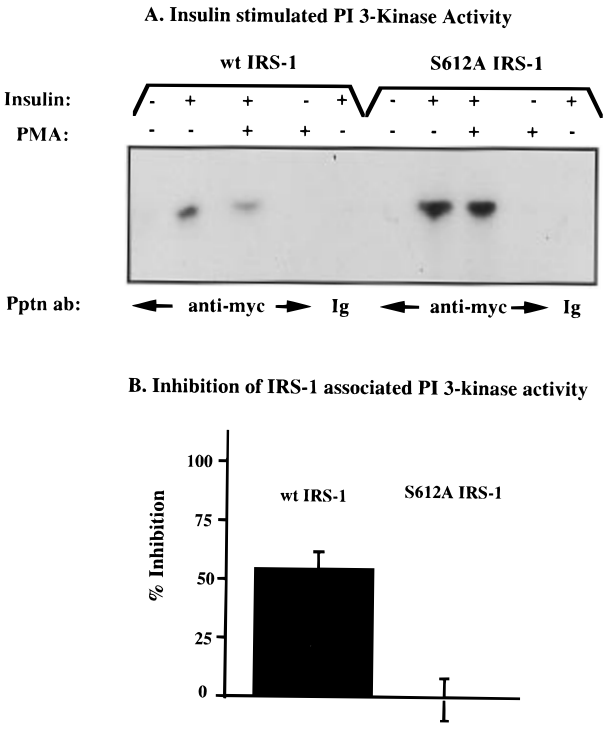

FIGURE 3: Effect of PKC activation on the insulin-induced association of PI 3-kinase with wild-type and S612A mutant IRS-1. (A) IRS-1-associated PI 3-kinase activity. Cells were pretreated with PMA as indicated and then stimulated with insulin. Cell lysates were immunoprecipitated (Pptn) with either anti-myc antibodies or control Ig, as indicated. The beads were washed, and the amount of bound PI 3-kinase activity was determined. (B) Quantitation of the IRS-1-associated PI 3-kinase activity. The results from four experiments were pooled, and the extent of inhibition by PMA pretreatment was calculated for the wild-type (wt) and mutant IRS-1.

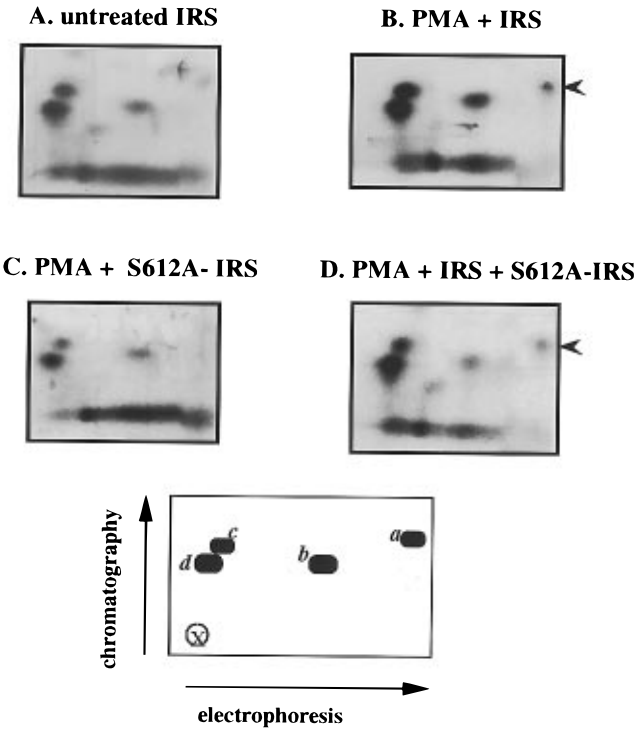

FIGURE 4: Tryptic peptide mapping of *in vivo* labeled IRS-1 and S612A mutant IRS-1 from untreated and PMA-treated 293 cells. Cells were labeled with [^{32}P]orthophosphate for 12−15 h; PMA was added as indicated during the last 20 min of labeling. Cell lysates were immunoprecipitated with anti-myc antibodies; the precipitates were analyzed by SDS−PAGE, and proteins were transferred to Immobilon-P membranes. Radiolabeled IRS-1 was visualized by autoradiography, and bands were excised and subjected to trypsin digestion. Trypsinized samples were spotted onto TLC plates and analyzed in two dimensions, by electrophoresis in pH 1.9 buffer followed by chromatography in phosphochromatography buffer, as indicated. Maps of wt IRS-1 from untreated cells (A) and PMA-treated cells (B), S612 mutant IRS-1 from PMA-treated cells (C), and a mixture of the mutant and wt IRS-1 from PMA-treated cells (D) are shown, and a legend to the individual spots is depicted. The spot corresponding to the peptide containing S612, spot a, is indicated by the arrow in panels B and D.

in the second dimension by chromatography. Peptide maps of wild-type IRS-myc show the presence of four distinct spots that increase in intensity upon addition of PMA (Figure 4A,B), described in the legend as a, b, c, and d. In contrast, the peptide maps of the S612A mutant IRS-1 from PMA-treated cells lack spot a, but contain spots b, c, and d (Figure 4C). When tryptic digests of the wild-type and mutant IRS-1 are mixed, spots b, c, and d from the two proteins are found to comigrate, demonstrating that only peptide a is unique to the wild-type IRS-1 (Figure 4D)

Tyrosine Phosphorylation of an IRS-1 Peptide Is Inhibited by Phosphorylation on Serine 612 Equivalent Residue. Isolated IR can phosphorylate *in vitro* a peptide (KKHTDDGYMPMSPGVA) that corresponds to amino acid residues 604−616 of IRS-1 with the addition of two extra amino-terminal basic residues (Boge & Roth, 1995). To determine whether a phosphoserine at the serine 612 equivalent position of IRS-1 would affect the tyrosine phosphorylation of this peptide, the phosphoserine peptide (KKHTDDGYMPMpSPGVA) was synthesized and compared for its ability to be phosphorylated *in vitro* by isolated IR. Various concentrations of the wild-type or phosphoserine peptide were incubated with isolated IR in the presence of [γ-^{32}P]ATP. Phosphorylation of the peptides was analyzed either by electrophoresis on polyacrylamide gels (Figure 5A) or by spotting onto phosphocellulose. Both procedures showed that the extent of phosphorylation of the phosphopeptide was much less than the nonphosphorylated peptide (Figure 5). Lineweaver−Burk plots of the data indicated that the K_m of the IR for the two peptides was comparable while the V_{max} for the phosphopeptide was reduced from that of the wild-type peptide. A time course of IR phosphorylation of the peptide indicates that the reaction time used for these experiments is within the linear range (data not shown). These results suggested that the phosphopeptide might serve to inhibit the phosphorylation of wild-type peptide by the IR kinase. Isolated IR was therefore incubated with a fixed concentration of the nonphosphorylated peptide (500 μM) in the presence of increasing concentrations of the phosphopeptide. The phosphopeptide clearly inhibited the phosphorylation of the nonphosphorylated peptide with an IC_{50} of about 150 μM, a value close to the K_m of the peptide (Figure 5C).

PMA Activates a Cytosolic Kinase Activity Capable of Phosphorylating the IRS-1 Peptide and IRS-1. To test whether activation of PKC stimulates the enzymatic activity of a kinase capable of phosphorylating IRS-1, cytosol was prepared from either untreated or PMA-treated 293 cells. This cytosolic preparation was incubated with IRS-1 immunoprecipitated from 293-IRS cells in the presence of [γ-^{32}P]-ATP for 30 min at room temperature, and analyzed by SDS−PAGE followed by autoradiography. PMA pretreatment

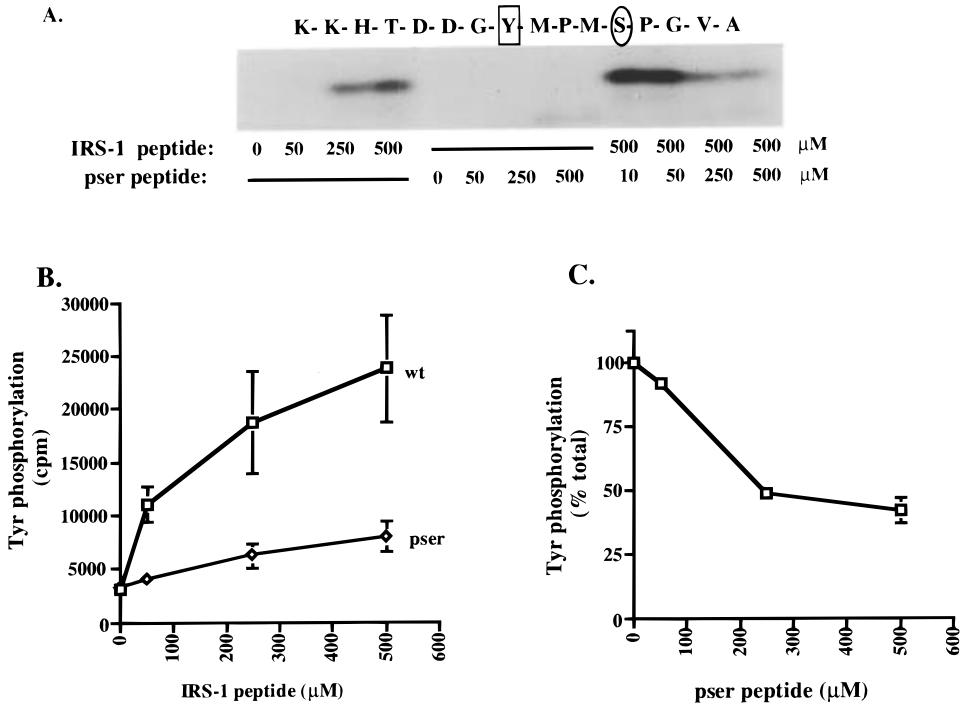

FIGURE 5: IR phosphorylation *in vitro* of an IRS-1 peptide with or without Ser phosphorylation. (A) IR prepared from 293 cells was activated *in vitro* and incubated with 0, 50, 250, or 500 μM of either the shown wild-type IRS-1 peptide, the same peptide with a phosphoserine (pser peptide), or a mixture of the two peptides. Samples were run on a 40% polyacrylamide gel, and a picture of the autoradiogram is shown. (B) Quantitation of *in vitro* phosphorylation of IRS-1 peptides. Peptide phosphorylation was quantified either by cutting out and counting the bands on the 40% gel or by spotting reactions onto phosphocellulose and counting. The results of four experiments were pooled and graphed as the number of counts incorporated into the peptide. (C) Inhibition of IRS-1 peptide phosphorylation by phosphoserine-containing peptide. IR was incubated with 500 μM wild-type peptide in the presence of 0, 10, 50, 250, and 500 μM phosphoserine peptide, and phosphorylation was quantitated as described above. Results are expressed as the percent of counts present in the absence of the pser peptide (100%).

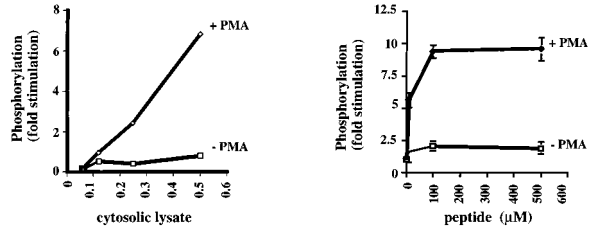

FIGURE 6: PMA-activated cytosolic kinase activity phosphorylates an IRS-1 peptide. Kinase reactions were performed with the indicated amounts of cytosolic lysate (expressed as a fraction of a 100 mm dish) from PMA-treated (+PMA) or control cells (−PMA) and 100 μM of the biotinylated IRS-1 peptide. Peptide was captured on streptavidin−agarose beads and counted or electrophoresed and cut out and counted. Similarly, the indicated amounts of peptide were incubated with cytosol prepared from PMA-treated and control cells. The results from five experiments were pooled and shown as a fold increase in phosphorylation with increaing peptide concentrations.

stimulated an enzymatic activity capable of phosphorylating IRS-1 (data not shown). To further characterize this enzymatic activity from PMA-treated cells, the cytosols were also tested for their ability to phosphorylate a biotinylated and a nonbiotinylated peptide corresponding to residues 604−616 of IRS-1. PMA pretreatment caused a 10-fold increase in an enzymatic activity capable of phosphorylating the IRS-1 peptide (Figure 6). This enzymatic activity was linearly dependent upon the amount of cytosolic extract added to the peptide and exhibited a K_m of about 10 μM for the peptide (Figure 6).

A Cytosolic Kinase Activity Capable of Phosphorylating the IRS-1 Peptide Is Found in the Livers of ob/ob Mice. To determine whether serine phosphorylation of IRS-1 might play a role in the physiological insulin resistance observed in animal models, we wished to determine whether a similar IRS-1 kinase activity was elevated in an insulin target tissue of *ob/ob* mice (Heydrick et al., 1993). We prepared cytosolic extracts from the livers of both *ob/ob* mice and their lean littermates, and these cytosolic lysates were incubated with the IRS-1 peptide described above in the presence of [γ-^{32}P]-ATP for 30 min at room temperature. Results from 4 experiments and a total of 22 mice were pooled, and the average kinase activity in *ob/ob* compared to lean mice was determined. The liver extracts from the lean mice incorporated approximately 840 $\pm$ 340 (n = 11) cpm into the peptide whereas the extracts from the livers of the obese mice incorporated 8800 $\pm$ 3300 cpm (n = 11) (Figure 7A). In the four experiments, the increase in IRS-1 kinase activity in *ob/ob* as compared to their lean littermates varied from 6- to 14-fold (Figure 7B).

DISCUSSION

In the present studies, an epitope-tagged version of IRS-1 has been stably expressed in human kidney fibroblast (293 cells) and shown to exhibit insulin-stimulated tyrosine phosphorylation and association with PI 3-kinase. The use of an epitope-tagged version of IRS-1 allows one to distinguish between the endogenous IRS-1 and the expressed form of this protein. Most important for the present studies, activation of PKC by PMA in these cells was found to inhibit the insulin-stimulated tyrosine phosphorylation of IRS-1 and its subsequent association with the PI 3-kinase. This system therefore differs from prior studies in Chinese hamster ovary

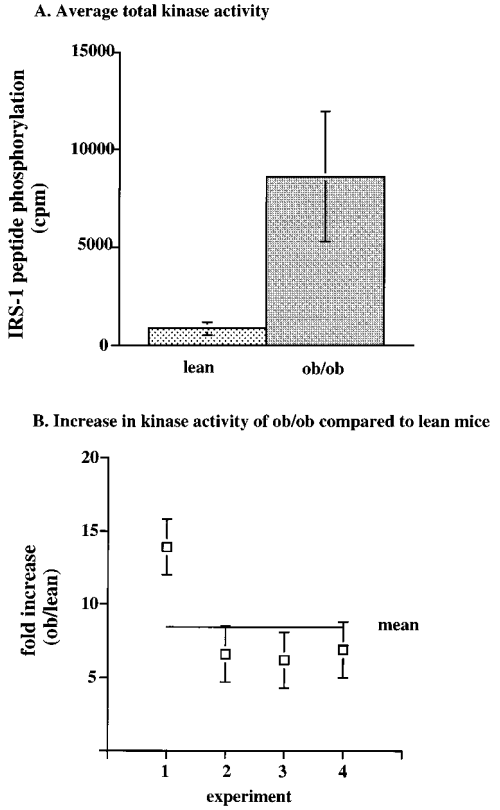

FIGURE 7: Cytosolic kinase activity capable of phosphorylating the IRS-1 peptide is increased in the livers of *ob/ob* mice as compared with their lean littermates. *Ob/ob* mice and their lean littermates, approximately 20−35 weeks old, were euthanized and the livers removed. Cytosolic extracts were prepared and incubated with either 250 μM of the biotinylated or the unbiotinylated peptide in the presence of [γ-^{32}P]ATP for 30 min at room temperature. Phosphorylated peptide was measured either by capturing on streptavidin−agarose beads and counting or by analysis on a 40% polyacrylamide gel, autoradiography, excision of bands, and counting. The results of 4 experiments and 22 total mice (11 *ob/ob* and 11 lean) were pooled, and the mean phosphorylation for each group was calculated (A). The fold increase in activity observed in *ob/ob* compared to lean mice in each experiment was calculated, and and the mean increase for all 4 experiments was determined (B).

cells in which it was necessary to overexpress PKC to observe an inhibition of insulin signaling (Chin et al., 1993, 1994). The requirement for overexpression of PKC in the CHO cells may in part be due to the overexpression of the IR in this system whereas in the present studies only the endogenous receptors were present (Chin et al., 1994). It is also possible that there are differences in the particular PKC isoforms present in the two cell types or to the presence of a distinct serine kinase capable of phosphorylating IRS-1 in response to PKC activation in the 293 cells. In preliminary studies, PKCθ was found to be present in 293 cells but not Chinese hamster ovary cells. Moreover, attempts to phosphorylate the IRS-1 peptide with extracts prepared from PMA-treated CHO-PKCα cells were less successful than with the 293 cells.

We then tested whether a particular serine, serine 612, in the IRS-1 molecule was required for the ability of PKC to modulate the insulin-stimulated tyrosine phosphorylation of this protein. This serine was chosen since it is in close proximity to tyrosine 608, a residue known to be phosphorylated by the IR and to play a major role in PI 3-kinase binding (Rocchi et al., 1995; Sun et al., 1991). This serine was changed to alanine, and stable cell lines expressing this mutant IRS-1 molecule were established. This mutant IRS-1 was expressed to a similar extent as the wild-type IRS-1, did not exhibit increased degradation, and exhibited a comparable level of insulin-stimulated tyrosine phosphorylation as the wild-type IRS-1, all indicating that there was no gross conformational change in the molecule as a result of the introduction of this mutation. This mutation did, however, eliminate the ability of PKC to negatively modulate both the *in situ* and *in vitro* tyrosine phosphorylation of IRS-1. Furthermore, this mutation also eliminated the ability of PKC to negatively modulate the insulin-stimulated association of PI 3-kinase with IRS-1 (Figures 1−3). These result indicate that serine 612 plays a critical role in allowing the activation of PKC to modulate the interaction of IRS-1 with the IR. Moreover, tryptic peptide mapping of *in vivo* labeled wild-type and S612A mutant IRS-1 demonstrates that PKC activation results in the phosphorylation of a peptide in the wild-type IRS-1 that is missing in the S612 mutant, the S612-containing peptide. It is actually quite surprising that the mutation of this single serine has such a pronounced effect on the tyrosine phosphorylation of IRS-1. It is possible that phosphorylation at this site may affect subsequent serine phosphorylations of IRS-1, possibly via a kinase which requires prior phosphorylation at this site (for example, via an enzyme like glycogen synthase kinase-3).

A number of distinct models may explain the observed data. The simplest interpretation is that activation of PKC stimulates a ser/thr kinase which then phosphorylates IRS-1 at S612 and possibly other residues, and this phosphorylation inhibits the subsequent ability of the IRS-1 to serve as a substrate for the IR tyrosine kinase. Since serine 612 is not in a motif which is known to be directly phosphorylated by PKC (Kennelly & Krebs, 1991), it would likely be a distinct kinase activated by PKC which is phosphorylating this residue. To further test this hypothesis, a number of subsequent experiments were performed.

We first examined whether PMA treatment of the 293 cells stimulated the enzymatic activity of a kinase which phosphorylates IRS-1. A cytosolic kinase was identified in the extracts of 293 cells whose ability to phosphorylate intact IRS-1 was stimulated by PMA treatment. PMA treatment of the 293 cells was also found to cause a 10-fold increase in the enzymatic activity of a cytosolic kinase which could phosphorylate a synthetic peptide based on the IRS-1 sequence (Figure 6). This peptide contains only a single serine, the serine 612 equivalent residue of IRS-1. It is possible, however, that other serines in IRS-1 are also involved in modulating its ability to be tyrosine phosphorylated by the IR. In a prior study, the replacement of serine 612 with alanine did not decrease the ability of okadaic acid to inhibit the insulin-stimulated tyrosine phosphorylation of IRS-1 (Mothe & Van Obberghen, 1996). This difference may be due to the ability of okadaic acid to cause the stimulation of the enzymatic activities of multiple serine/threonine kinases by inhibiting serine/threonine phosphatases. Thus, additional serines in IRS-1 may also be phosphorylated and regulate its ability to interact with the IR. It will be of interest to determine whether serine 612 participates in the ability of other factors (i.e., tumor necrosis factor, growth hormone, etc.) to modulate the insulin-stimulated tyrosine phosphorylation of IRS-1. It is also possible that the serine phosphorylation of IRS-1 acts synergistically with the serine

phosphorylation of the IR to inhibit the insulin-stimulated tyrosine phosphorylation of IRS-1.

To further test the above model, a synthetic peptide with phosphoserine at the equivalent position of serine 612 was synthesized and examined for its ability to be phosphorylated by the isolated IR. We found that the phosphopeptide was a much poorer substrate *in vitro* for the IR kinase than the equivalent peptide which was not phosphorylated at this position (Figure 5). Moreover, this phosphopeptide actually served as an inhibitor of the IR kinase. It is therefore possible that IRS-1 phosphorylated at serine 612 could also serve as an inhibitor of the IR kinase. Recent studies have in fact suggested that tumor necrosis factor induces an increase in the serine phosphorylation of IRS-1 and this serine-phosphorylated molecule was reported to inhibit the IR kinase (Hotamisligil et al., 1996).

Finally, to determine whether our observations in the 293 cells were applicable to a more physiological system of insulin resistance (Heydrick et al., 1993), we examined tissues from *ob/ob* mice for the presence of the IRS-1 serine kinase. Extracts from livers of *ob/ob* mice exhibited a greatly increased ability to phosphorylate this peptide as compared to their lean littermates (Figure 7). These results are therefore consistent with the hypothesis that an excessive serine phosphorylation of IRS-1 is occurring in these animals, possibly due to the presence of activated PKC in these tissues.

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