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Protein Kinase C-ζ Phosphorylates Insulin Receptor Substrate-1 and Impairs Its Ability to Activate Phosphatidylinositol 3-Kinase in Response to Insulin*

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Lingamanaidu V. Ravichandran, Diana L. Esposito‡, Judy Chen, and Michael J. Quon§

From the Cardiology Branch, NHLBI, National Institutes of Health, Bethesda, Maryland 20892 and the ‡Department of Oncology and Neurosciences, University "G. D'Annunzio," Chieti 66013, Italy

Protein kinase C-ζ (PKC-ζ) is a serine/threonine kinase downstream from phosphatidylinositol 3-kinase in insulin signaling pathways. However, specific substrates for PKC-ζ that participate in the biological actions of insulin have not been reported. In the present study, we identified insulin receptor substrate-1 (IRS-1) as a novel substrate for PKC-ζ. Under *in vitro* conditions, wild-type PKC-ζ (but not kinase-deficient mutant PKC-ζ) significantly phosphorylated IRS-1. This phosphorylation was reversed by treatment with the serine-specific phosphatase, protein phosphatase 2A. In addition, the overexpression of PKC-ζ in NIH-3T3^{IR} cells caused significant phosphorylation of cotransfected IRS-1 as demonstrated by [^{32}P]orthophosphate labeling experiments. In rat adipose cells, endogenous IRS-1 coimmunoprecipitated with endogenous PKC-ζ, and this association was increased 2-fold upon insulin stimulation. Furthermore, the overexpression of PKC-ζ in NIH-3T3^{IR} cells significantly impaired insulin-stimulated tyrosine phosphorylation of cotransfected IRS-1. Importantly, this was accompanied by impaired IRS-1-associated phosphatidylinositol 3-kinase activity. Taken together, our results raise the possibility that IRS-1 is a novel physiological substrate for PKC-ζ. Because PKC-ζ is located downstream from IRS-1 and phosphatidylinositol 3-kinase in established insulin signaling pathways, PKC-ζ may participate in negative feedback pathways to IRS-1 similar to those described previously for Akt and GSK-3.

PKC-ζ[1] is an atypical member of the protein kinase C family of Ser/Thr kinases that is an important mediator of the metabolic actions of insulin, such as translocation of the insulin-responsive glucose transporter GLUT4 and enhancement of glucose transport (1–4). Activation of PKC-ζ in response to insulin stimulation is regulated by PI 3-kinase-dependent pathways and involves PDK-1 phosphorylating Thr^{410} in the PKC-ζ activation loop (5, 6). Known substrates of PKC-ζ include nucleolin (7), heterogeneous ribonucleoprotein A1 (8), Sp1 (9), Sendai virus phosphoprotein (10), and IKKβ (11). However, direct substrates of PKC-ζ that participate in insulin signaling have not been identified to date.

IRS-1 is a substrate of the insulin receptor tyrosine kinase that upon tyrosine phosphorylation functions as a docking molecule to engage and activate SH2 domain-containing proteins, including PI 3-kinase and SHP-2 (12). IRS-1 is also extensively phosphorylated on serine residues, and modulation of IRS-1 serine phosphorylation is observed in response to the treatment of cells with a variety of agents, including insulin (13, 14), tumor necrosis factor α (15–17), PDGF (18), angiotensin II (19), phorbol esters (20, 21), and okadaic acid (14, 22). Interestingly, IRS-1 has recently been identified as a substrate for both Akt and GSK-3, Ser/Thr kinases downstream from PI 3-kinase that participate in the metabolic actions of insulin (23, 24). These studies suggest that feedback mechanisms in metabolic insulin signaling pathways may exist at the level of IRS-1 to regulate insulin sensitivity (23, 24). Although serine phosphorylation of IRS-1 has generally been associated with impairment in its ability to couple with PI 3-kinase in response to insulin (14, 16–19, 21, 22, 24, 25), phosphorylation of IRS-1 by Akt may also positively modulate its function (23). In the present study, we found that IRS-1 may also be a novel physiological substrate for PKC-ζ that participates in the feedback regulation of metabolic insulin signaling pathways.

MATERIALS AND METHODS

Reagents

Reagents were obtained from the following sources: monoclonal anti-HA antibody from Covance Research Products (Denver, PA); monoclonal anti-phosphotyrosine antibody (4G10), polyclonal anti-p85, anti-IRS-1, and anti-PDGFβ receptor antibodies, recombinant human IRS-1 protein, and recombinant protein phosphatase 2A from Upstate Biotechnology, Inc. (Lake Placid, NY); recombinant PKC-ζ from PanVera Corporation (Madison, WI); goat polyclonal antibodies against N-terminal and C-terminal regions of PKC-ζ, sheep polyclonal anti-SHP-2 antibody, and rabbit polyclonal anti-HA antibody from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); and protein A- and protein G-agarose beads and LipofectAMINE Plus from Life Technologies, Inc.

Expression Plasmids

pCIS2—This is the parental expression vector with a cytomegalovirus promoter (26, 27).

IRS1-HA—cDNA for human IRS-1 was subcloned into pCIS2 as described (28), and the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) was used to create sequence coding for an HA-epitope tag fused to the C terminus of IRS-1 (sense primer, 5′-G CAG CCA GAG GAC CGT CAG TAT CCT TAT GAT GTT CCT GAT TAT GCT TAG CTC AAC TGG ACA TCA CAG C-3′; antisense primer, 5′-G CTG TGA TGT CCA GTT GAG CTA AGC ATA ATC AGG AAC ATC ATA AGG ATA CTG ACG CTC CTC TGG CTG C-3′).

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§ To whom correspondence should be addressed: Cardiology Branch, NHLBI, National Institutes of Health, Bldg. 10, Rm. 8C-218, 10 Center Dr. MSC 1755, Bethesda, MD 20892-1755. Tel.: 301-496-6269; Fax: 301-402-1679; E-mail: quonm@nih.gov.

[1] The abbreviations used are: PKC-ζ, protein kinase C-ζ; WT, wild type; KD, kinase-deficient; PI, phosphatidylinositol; IRS-1, insulin receptor substrate-1; SH, Src homology; PDGF, platelet-derived growth factor; HA, hemagglutinin; PAGE, polyacrylamide gel electrophoresis; TES, 2-{[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino}ethanesulfonic acid; GSK-3, glycogen synthase kinase-3.

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IRS1-Y612/Y632—This construct was derived from IRS1-HA by using the QuikChange kit to sequentially introduce Phe for Tyr substitutions in YXXM motifs at positions 465, 662, 941, and 989 with the mutagenic oligonucleotides: 465 sense, 5′-G CTA AGC AAC TTT ATC TGC ATG GGT GGC-3′; 465 antisense, 5′-GCC ACC CAT GCA GAT AAA GTT GCT TAG C-3′; 662 sense, 5′-TG GAC CCC AAT GGC TTC ATG ATG ATG TCC-3′; 662 antisense, 5′-GGA CAT CAT CAT GAA GCC ATT GGG GTC CA-3′; 941 sense, 5′-GGC ACT GAG GAG TTC ATG AAG ATG GAC C-3′; 941 antisense, 5′-G GTC CAT CTT CAT GAA CTC CTC AGT GCC-3′; 989 sense, 5′-AGC CGG GGT GAC TTC ATG ACC ATG CAG-3′; 989 antisense; 5′-CTG CAT GGT CAT GAA GTC ACC CCG GCT-3′. This mutagenesis left YXXM sites at Tyr^{612} and Tyr^{632} intact.

PKCζ-WT—This is the rat wild type PKC-ζ with an N-terminal HA-epitope tag in the pCDNA3 expression vector (29, 30).

PKCζ-KD—This is the kinase-inactive point mutant of rat PKC-ζ (L281W) with N-terminal HA-epitope tag in pCDNA3 expression vector (29, 30).

Cell Culture and Transfection

NIH-3T3 fibroblasts stably overexpressing human insulin receptors ($NIH\text{-}3T3^{IR}$) were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, L-glutamine (2 mM), penicillin (100 units/ml), and streptomycin (100 μg/ml) in a humidified atmosphere with 5% CO_2 at 37 °C. $NIH\text{-}3T3^{IR}$ cells were transiently transfected with PKC-ζ and/or IRS-1 constructs using LipofectAMINE Plus according to the manufacturer's instructions. Rat adipose cells were obtained by collagenase digestion of epididymal fat pads of male rats as described (27) and were used within 2 h of isolation.

In Vitro PKC-ζ Kinase Assays

In vitro kinase assays using PKC-ζ and IRS-1 were carried out at 30 °C for 30 min in kinase assay buffer containing 50 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, 50 μM ATP, 2.5 μCi of [γ-^{32}P]ATP/assay, and 4 μg of phosphatidylserine. The reactions were stopped by adding Laemmli sample buffer and boiling for 10 min. Samples were subjected to 7.5% SDS-PAGE, and phosphorylated IRS-1 was detected with a PhosphorImager. In addition, gel contents were transferred to nitrocellulose and immunoblotted with anti-IRS-1 antibody. Finally, the activity of PKC-ζ in each assay was independently verified using peptide ε as a substrate (31). For assays using purified PKC-ζ and IRS-1 proteins, 0.5 μg of IRS-1 and 0.1 μg of PKC-ζ (specific activity of, 1410 nmol of phosphate transferred to peptide ε substrate/min/mg of protein) were used. In some experiments, endogenous IRS-1 or recombinant PKC-ζ was immunoprecipitated from lysates of $NIH\text{-}3T3^{IR}$ cells (1 mg of total protein) prepared using lysis buffer (50 mM Tris-HCl, pH 7.4, 125 mM NaCl, 1% Triton X-100, 0.5% Nonidet P-40, 1 mM Na_3VO_4, 50 mM NaF, 1 mM sodium pyrophosphate, 10 mM β-glycerophosphate, 0.1 mM okadaic acid, and a complete protease inhibitor mixture (Roche Molecular Biochemicals)). Lysates precleared with protein G-agarose beads for 1 h at 4 °C were immunoprecipitated with antibodies against the HA-epitope (to obtain HA-tagged PKC-ζ) or IRS-1 and protein G-agarose beads for 2 h at 4 °C. The immunocomplexes were then washed four times with kinase assay buffer and used for *in vitro* kinase assays as described above.

In Vivo Phosphorylation Experiments

$NIH\text{-}3T3^{IR}$ cells transiently transfected with HA-tagged IRS-1 and PKC-ζ constructs were serum-starved overnight and then labeled for 2 h with 75 μCi/ml [^{32}P]orthophosphate in KRBH buffer, pH 7.4 (107 mM NaCl, 5 mM KCl, 3 mM $CaCl_2$, 1 mM $MgSO_4$, 20 mM Hepes, 10 mM glucose, 1% bovine serum albumin, and 7 mM $NaHCO_3$). Cells were then washed four times with phosphate-buffered saline, and cell lysates were subjected to immunoprecipitation with anti-HA antibody and protein A-agarose beads. Immunocomplexes were washed four times with lysis buffer, boiled in Laemmli sample buffer, and separated by 7.5% SDS-PAGE. Phosphorylated IRS-1 was detected and quantified with a PhosphorImager. Gel contents were transferred to nitrocellulose for immunoblotting with anti-IRS-1 antibody.

Coimmunoprecipitation Experiments

Using an anti-IRS-1 antibody, IRS-1 was immunoprecipitated from cell lysates (500 μg of total protein) derived from $NIH\text{-}3T3^{IR}$ cells transiently transfected with the empty control vector, PKCζ-WT or PKCζ-KD, as described above. Cell lysates were precleared with protein G-agarose beads to minimize nonspecific binding. As an additional control for nonspecific binding, samples were also immunoprecipitated with preimmune rabbit IgG. Both cell lysates and immunoprecipitated samples were immunoblotted with antibodies against IRS-1 and the HA-epitope (to detect HA-tagged PKC-ζ). The coimmunoprecipitation of endogenous IRS-1 and PKC-ζ was examined in freshly isolated rat adipose cells stimulated without or with insulin (100 nM, 5 min). Cell lysates were prepared as described previously using ice-cold TES buffer (containing 1% Triton X-100, 0.5% Nonidet P-40, and inhibitors as listed above for lysis buffer) (32). Anti-PKC-ζ antibodies directed against either the N- or C-terminal regions of PKC-ζ were used to immunoprecipitate endogenous PKC-ζ. As an additional control for nonspecific binding, samples were also immunoprecipitated with preimmune goat IgG. Both cell lysates and immunoprecipitated samples were immunoblotted with antibodies against IRS-1 and PKC-ζ.

Functional Assessment of IRS-1

$NIH\text{-}3T3^{IR}$ cells transiently cotransfected with IRS1-HA and either PKCζ-WT or a control vector were serum-starved overnight and then stimulated with insulin (100 nM) for 0, 2, or 60 min. Cell lysates (300–500 μg of total protein) were subjected to immunoprecipitation with anti-HA antibody as described above and separated by 7.5% SDS-PAGE, and gel contents were transferred to nitrocellulose. Membranes were immunoblotted with antibodies against IRS-1, p85, and SHP-2 and then stripped and reprobed with phosphotyrosine antibody. Cell lysates from each group were also immunoblotted for IRS-1 and PKC-ζ. To assess IRS-1-associated PI 3-kinase activity, anti-HA immunoprecipitates were washed once with phosphate-buffered saline containing 1% Nonidet P-40 and 100 μM Na_3VO_4, twice with 100 mM Tris-HCl, pH 7.5, containing 500 mM $LiCl_2$ and 100 mM Na_3VO_4, and once with 10 mM Tris-HCl, pH 7.5, containing 100 mM NaCl, 1 mM EDTA, and 100 mM Na_3VO_4. For each reaction, 10 μg of phosphatidylinositol (Sigma) sonicated in 10 μl of PI 3-kinase reaction buffer (20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 0.3 mM EGTA) and 10 μCi of [γ-^{32}P]ATP in 40 μl of PI 3-kinase reaction buffer were added along with $MgCl_2$ at a final concentration of 10 mM. The phosphorylation reaction was started by adding 50 μl of the substrate solution with 50 μl of the immune complex. After incubation for 20 min at 30 °C, the reaction was stopped by adding 100 μl of 0.1 N HCl and 200 μl of $CHCl_3/CH_3OH$ (1:1). The organic phase containing the phosphorylated phospholipid product was extracted and applied to a silica gel thin layer chromatography plate (Whatman) coated with 1% potassium oxalate. Thin layer chromatography plates were developed in $CHCl_3/CH_3OH/H_2O/NH_4OH$ (60:47:11.3:2), dried, and visualized by autoradiography. Assays were quantified by PhosphorImager and normalized for the amount of IRS-1 recovered in anti-HA immunoprecipitates.

RESULTS

IRS-1 Is a Substrate for PKC-ζ Both in Vitro and in Intact Cells—*In vitro* kinase assays using purified PKC-ζ and IRS-1 proteins were carried out to determine whether IRS-1 is capable of functioning as a substrate for PKC-ζ. Incubation of PKC-ζ with IRS-1 in the presence of [^{32}P]ATP significantly increased the amount of phosphorylated IRS-1 (Fig. 1, *lanes 1* and *2*). This phosphorylation was substantially reversed upon treatment with protein phosphatase 2A (a Ser/Thr-specific phosphatase), suggesting that IRS-1 is phosphorylated on Ser/Thr residues by PKC-ζ (Fig. 1, *lane 3*). Endogenous IRS-1 immunoprecipitated from $NIH\text{-}3T3^{IR}$ cells was also specifically phosphorylated in the presence of PKC-ζ (Fig. 1, *lanes 4* and *5*). In addition, only wild-type PKC-ζ but not kinase-inactive mutant PKC-ζ (immunoprecipitated from transfected $NIH\text{-}3T3^{IR}$ cells) was able to phosphorylate purified IRS-1 *in vitro* (Fig. 1, *lanes 6* and *7*). We next performed *in vivo* labeling experiments in $NIH\text{-}3T3^{IR}$ cells transiently cotransfected with IRS1-HA and an empty expression vector (pCIS2), PKCζ-WT, or PKCζ-KD to determine whether IRS-1 can also function as a substrate for PKC-ζ in intact cells. Importantly, the overexpression of PKC-ζ led to a significant ~2.5-fold increase in phosphorylation of IRS1-HA (Fig. 2). By contrast, the overexpression of the kinase-inactive mutant PKCζ-KD did not result in increased phosphorylation of IRS1-HA (Fig. 2, *lane 3*). Taken together, these results raise the possibility that IRS-1 may be a novel physiological substrate for PKC-ζ.

Association between IRS-1 and PKC-ζ in Intact Cells—To

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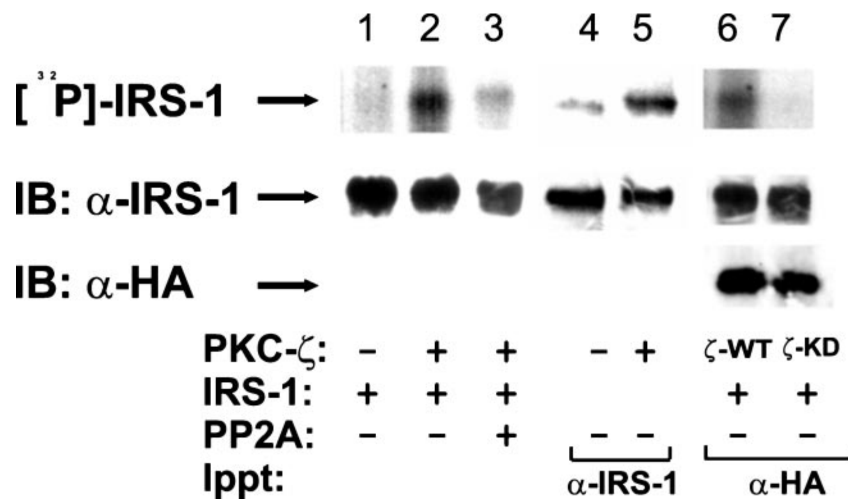

FIG. 1. **PKC-ζ phosphorylates IRS-1 *in vitro*.** Kinase assays were carried out *in vitro* in the presence of [γ-^{32}P]ATP using either purified PKC-ζ (*lanes 1–5*) or HA-tagged PKC-ζ constructs immunoprecipitated from lysates of transfected cells (*lanes 6* and *7*). Purified IRS-1 protein (*lanes 1–3*, *lanes 6* and *7*) or IRS-1 immunoprecipitated from cell lysates (*lanes 4* and *5*) were used as the substrate. *Top panel*, IRS-1 is phosphorylated by PKC-ζ (*lanes 2*, *5*, and *6*) but not by kinase-inactive mutant PKC-ζ (*lane 7*). Treatment with protein phosphatase 2A (30 min, 30 °C) reversed the phosphorylation of IRS-1 by PKC-ζ (*lane 3*). *Middle panel*, anti-IRS-1 immunoblot (*IB*) demonstrates comparable amounts of IRS-1 within each kinase assay. *Lower panel*, anti-HA immunoblot (*IB*) demonstrates comparable recovery of HA-tagged wild-type and mutant PKC-ζ (*lanes 6* and *7*). Representative results are shown for experiments that were independently repeated at least five times. *PP2A*, protein phosphatase 2A; *Ippt*, immunoprecipitate.

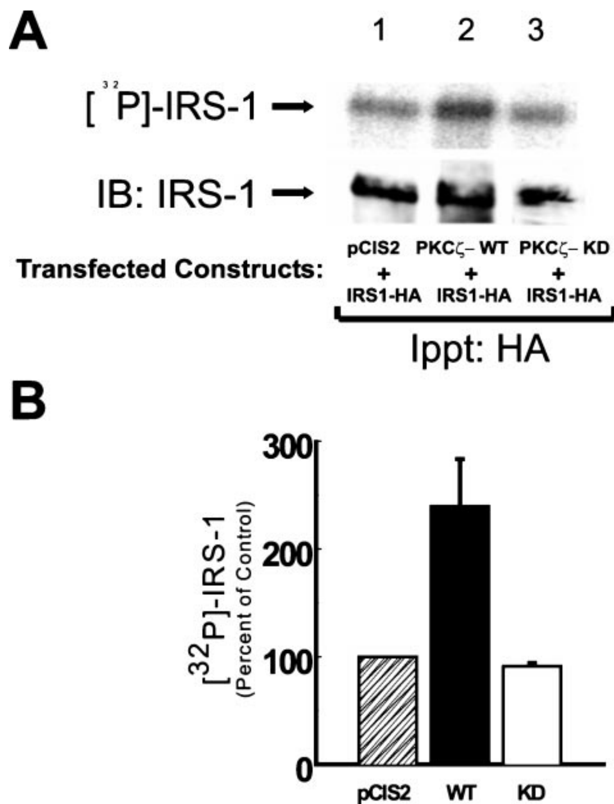

FIG. 2. **IRS-1 is a substrate for PKC-ζ in intact cells.** NIH-3T3^{IR} cells transiently transfected with HA-tagged IRS-1 and pCIS2 (empty vector), PKCζ-WT, or PKCζ-KD were labeled with [^{32}P]orthophosphate. Recombinant IRS-1 immunoprecipitated from cell lysates with anti-HA antibody was subjected to 7.5% SDS-PAGE and autoradiography. *A*, *top panel*, autoradiogram from a representative *in vivo* labeling experiment. *A*, *lower panel*, anti-IRS-1 immunoblot (*IB*) demonstrating comparable recovery of HA-tagged IRS-1 in each group. *B*, PhosphorImager quantification of three independent autoradiograms (mean ± S.E.). *Ippt*, immunoprecipitate.

provide further support for the hypothesis that IRS-1 is a direct substrate for PKC-ζ *in vivo*, we next investigated the ability of PKC-ζ to interact with IRS-1 in NIH-3T3^{IR} cells overexpressing either wild-type or kinase-inactive mutant PKC-ζ. Interestingly, both wild-type and mutant PKC-ζ coimmunoprecipitated with endogenous IRS-1 under our experimental conditions (Fig. 3). Neither wild-type nor mutant PKC-ζ was detected in control immunoprecipitation experiments performed with preimmune rabbit IgG (Fig. 3, *lanes 3* and *4*). We studied these interactions in a more physiological context by examining the ability of endogenous IRS-1 to coimmunoprecipitate with endogenous PKC-ζ in freshly isolated rat adipose cells. Lysates of adipose cells stimulated without or with insulin were immunoprecipitated using antibodies against PKC-ζ and then immunoblotted for both IRS-1 and PKC-ζ. Consistent with our results in NIH-3T3^{IR} cells, IRS-1 coimmunoprecipitated with PKC-ζ in the basal state (absence of insulin) (Fig. 4*A*, *lanes 1* and *3*). Upon insulin stimulation, we observed a significant ~2-fold increase in the amount of IRS-1 associated with PKC-ζ (Fig. 4, *A* (*lanes 2* and *4*) and *B*). Comparable results were obtained using antibodies directed against either the N- or C-terminal regions of PKC-ζ. No IRS-1 was detected in control immunoprecipitation experiments performed with preimmune goat IgG (Fig. 4*A*, *lanes 5* and *6*). These results suggest that regulated interactions between PKC-ζ and IRS-1 may potentially contribute to insulin action in *bona fide* insulin target cells.

Overexpression of PKC-ζ Impairs Insulin-stimulated Tyrosine Phosphorylation of IRS-1 and Associated PI 3-Kinase Activity—To assess the functional consequences of IRS-1 Ser/Thr phosphorylation by PKC-ζ, the time course of IRS-1 tyrosine phosphorylation after insulin stimulation was examined in NIH-3T3^{IR} cells transiently cotransfected with IRS1-HA and either a control vector or PKCζ-WT (Fig. 5). In control cells, insulin stimulation resulted in significant tyrosine phosphorylation of IRS1-HA by 2 min that decreased ~60% by 60 min (Fig. 5, *A* (*lanes 1–3*) and *B*). Interestingly, when compared with results from control cells, insulin-stimulated tyrosine phosphorylation of IRS1-HA recovered from cells overexpressing PKC-ζ was significantly reduced (Fig. 5*A*, *lanes 5* and *6*). Quantification of results from three independent experiments normalized for IRS1-HA recovery showed that the overexpression of PKC-ζ was associated with an ~40% decrease in tyrosine phosphorylation of IRS-1 at both the 2- and 60-min time points when compared with control cells (Fig. 5*B*). Thus, Ser/Thr phosphorylation of IRS-1 by PKC-ζ may negatively modulate IRS-1

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function by impairing insulin-stimulated tyrosine phosphorylation of IRS-1.

Because tyrosine phosphorylation of IRS-1 is required to engage and activate downstream SH2 domain-containing effectors, we studied the effects of the overexpression of PKC-ζ on insulin-stimulated interactions between IRS-1 and PI 3-kinase or SHP-2. NIH-3T3^{IR} cells transiently cotransfected with IRS1-HA and either a control vector or PKCζ-WT were stimulated with insulin, and anti-HA immunoprecipitates were immunoblotted for the p85 regulatory subunit of PI 3-kinase. As expected, insulin stimulation resulted in a significant increase in association between IRS1-HA and p85 in control cells (Fig. 6*A*, *top panel*, *lanes 1–3*). Interestingly, the overexpression of PKCζ-WT did not appear to alter the amounts of p85 associated with IRS1-HA in response to insulin when compared with controls (Fig. 6*A*, *top panel*, *lanes 4–6*). Nevertheless, when we examined PI 3-kinase activity in the anti-HA immunoprecipitates, the overexpression of PKCζ-WT was associated with a significant ~65% decrease in IRS-1-associated PI 3-kinase activity at the 0-, 2-, and 60-min time points when compared with the control group (Fig. 6, *A* (*middle panel*, *lanes 1–6*) and *B*). Thus, impairment in insulin-stimulated IRS-1 tyrosine phosphorylation caused by the overexpression of PKC-ζ was accompanied by a significant decrease in IRS-1-associated PI 3-kinase activity. The overexpression of PKC-ζ did not alter the amount of SHP-2 coimmunoprecipitated with IRS-1 in response to insulin stimulation (data not shown).

Human IRS-1 contains six tyrosine residues (Tyr^{465}, Tyr^{612}, Tyr^{632}, Tyr^{662}, Tyr^{941}, and Tyr^{987}) in Y*XX*M motifs that may bind the SH2 domains of p85 (12). We recently showed that the presence of Tyr^{612} and Tyr^{632} (in the absence of Tyr^{465}, Tyr^{662}, Tyr^{941}, and Tyr^{987}) is sufficient to mimic the ability of wild-type IRS-1 to activate PI 3-kinase and mediate translocation of GLUT4 in rat adipose cells whereas IRS-1 missing all six of these Y*XX*M motifs is unable to engage and activate PI 3-kinase (33). Therefore, we also examined the effects of the overexpression of PKC-ζ on the ability of IRS1-Y612/Y632 to associate with p85 and activate PI 3-kinase in response to insulin. Similar to results we obtained with wild-type IRS-1, the overexpression of PKC-ζ was not associated with alteration in p85 association with IRS1-Y612/Y632 but did cause significant impairment in IRS-1-associated PI 3-kinase activity at all time points (Fig. 6, *A* (*lanes 7–12*) and *B*). These results suggest that phosphorylation of IRS-1 by PKC-ζ specifically interferes with the function of Tyr^{612} or Tyr^{632}. To rule out the possibility that PKC-ζ was directly inhibiting PI 3-kinase activity, we compared PI 3-kinase activity in anti-PDGF receptor immunoprecipitates from cells cotransfected with PDGFβ receptor and either a control vector or PKCζ-WT. We did not observe any significant ability of PKC-ζ to impair PDGF-stimulated PI 3-kinase activity associated with the PDGF receptor (Fig. 7).

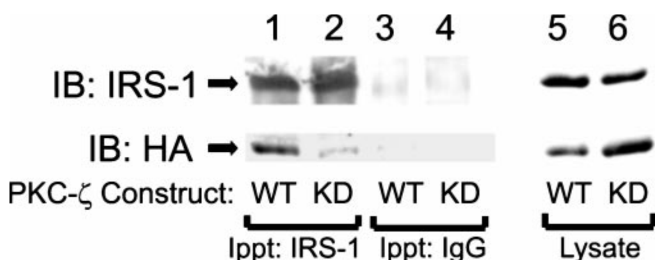

FIG. 3. **PKC-ζ coimmunoprecipitates with IRS-1 in transfected NIH-3T3^{IR} cells.** Lysates from cells transiently transfected with HA-tagged PKC-ζ constructs (*lanes 5* and *6*) were immunoprecipitated using an anti-IRS-1 antibody (*lanes 1* and *2*) or preimmune rabbit IgG as a control (*lanes 3* and *4*). In the representative blots shown, immunoprecipitations with the IRS-1 antibody and preimmune IgG were done concurrently on the same lysates and run in adjacent lanes on the same gel. *Top panel*, anti-IRS-1 immunoblot (*IB*) demonstrating comparable recovery of IRS-1 in both anti-IRS-1 immunoprecipitates and cell lysates. *Lower panel*, anti-HA immunoblot demonstrating coimmunoprecipitation of PKC-ζ with IRS-1 (*lanes 1* and *2*) and expression of PKC-ζ constructs in transfected cells (*lanes 5* and *6*). Representative results are shown for experiments that were independently repeated at least three times. *Ippt*, immunoprecipitate.

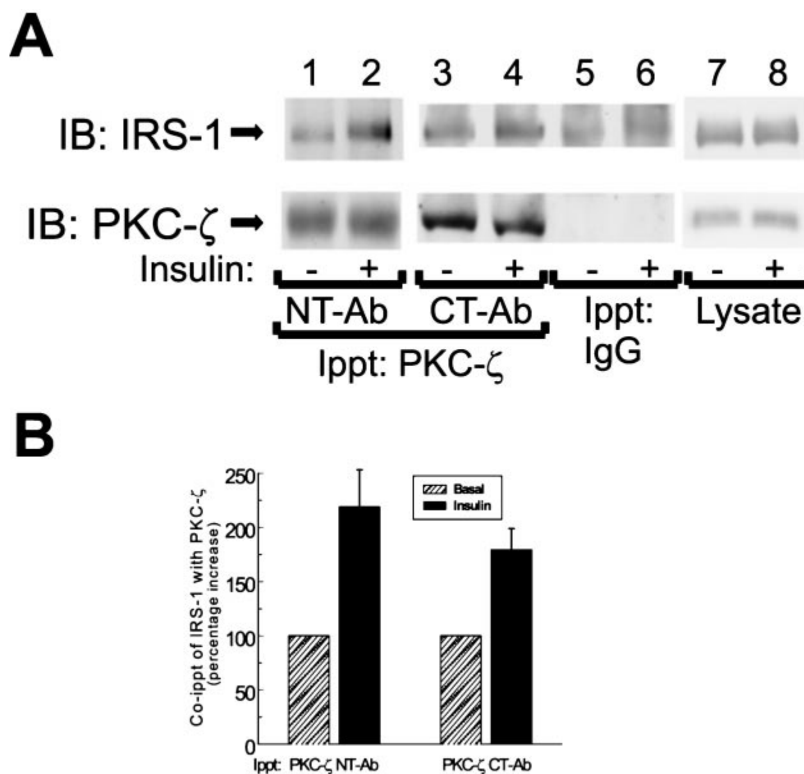

FIG. 4. **Association of IRS-1 with PKC-ζ in rat adipose cells is increased upon insulin stimulation.** Freshly isolated rat adipose cells were treated with insulin (100 nM, 5 min) as indicated, and cell lysates (*lanes 7* and *8*) were immunoprecipitated with antibodies against either N-terminal (*lanes 1* and *2*) or C-terminal (*lanes 3* and *4*) regions of PKC-ζ or preimmune goat IgG as a control (*lanes 5* and *6*). In the representative blots shown, immunoprecipitations with the C-terminal antibody (*CT-Ab*) and preimmune IgG were done concurrently on the same lysates and run in adjacent lanes on the same gel whereas the samples immunoprecipitated with the N-terminal antibody (*NT-Ab*) were run on a separate gel. *A*, *top panel*, anti-IRS-1 immunoblot (*IB*) demonstrating increased association between IRS-1 and PKC-ζ upon insulin stimulation. *A*, *lower panel*, anti-PKC-ζ immunoblot. *B*, quantification of IRS-1 coimmunoprecipitation (mean ± S.E.) based on scanning densitometry of four independent immunoblots normalized for PKC-ζ recovery. *Ippt*, immunoprecipitate.

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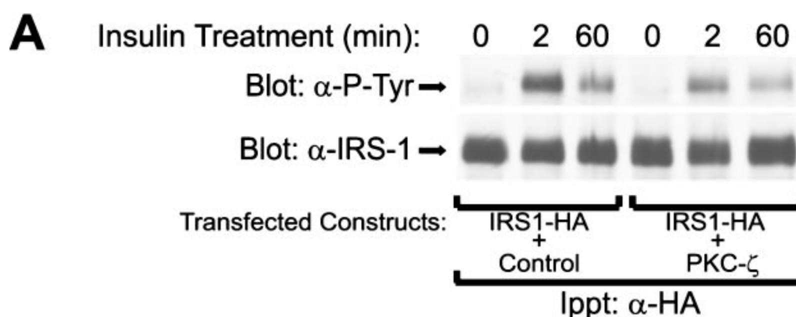


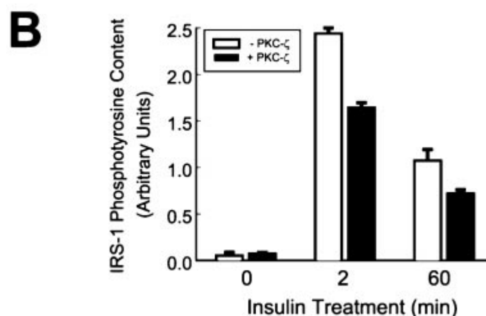
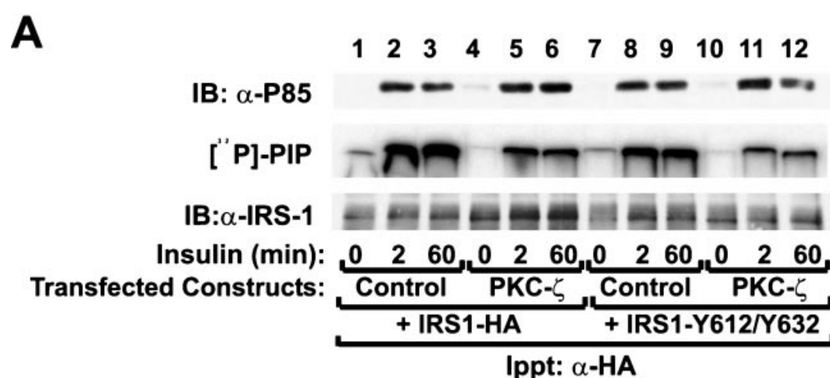


FIG. 5. **Overexpression of PKC-ζ in NIH-3T3^IR cells impairs insulin-stimulated tyrosine phosphorylation of IRS-1.** *A*, cells transiently transfected with HA-tagged IRS-1 and either a control vector or PKC-ζ were treated with insulin (100 nM) for 0, 2, or 60 min. Cell lysates were then immunoprecipitated with an anti-HA antibody followed by immunoblotting with an anti-phosphotyrosine antibody (*top panel*) or an anti-IRS-1 antibody (*lower panel*). *B*, quantification of IRS-1 phosphotyrosine content (mean ± S.E.) based on scanning densitometry of three independent immunoblots normalized for IRS-1 recovery. *Ippt*, immunoprecipitate.



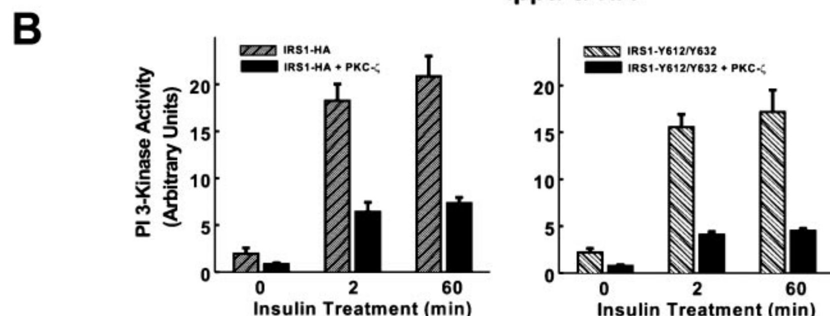


FIG. 6. **Effects of overexpression of PKC-ζ on the ability of IRS-1 to bind and activate PI 3-kinase.** NIH-3T3^IR cells transiently transfected with HA-tagged IRS-1 or IRS1-Y612/Y632 and either a control vector or PKC-ζ were treated with insulin (100 nM) for 0, 2, or 60 min. *A*, cell lysates were immunoprecipitated with anti-HA antibody followed by immunoblotting with anti-p85 antibody (*top panel*) or anti-IRS-1 antibody (*lower panel*). IRS-1-associated PI 3-kinase activity was assessed in a parallel set of HA immunoprecipitates by measuring the ^{32}P-labeled phosphatidylinositol phosphate (PIP) product (*middle panel*). *B*, PhosphorImager quantification of [^{32}P]phosphatidylinositol phosphate product. Results are mean ± S.E. of three independent experiments. *IB*, immunoblot; *Ippt*, immunoprecipitate.

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DISCUSSION

PKC-ζ is an effector of PI 3-kinase signaling pathways that plays an important role in metabolic actions of insulin (1–4). In addition, PKC-ζ mediates a number of other biological actions of insulin, including activation of p70 S6 kinase (34), ERK2 (35), and NHE1 (36) and stimulation of protein synthesis (37). However, downstream substrates of PKC-ζ in insulin signaling pathways have not been identified to date. Similarly, direct substrates of PKC-ζ upstream of PI 3-kinase that may be involved with feedback regulation of PKC-ζ have not previously been reported. Among the known substrates for PKC-ζ that are unrelated to insulin signaling (7–11), specific phosphorylation sites have only been identified for IKKβ (11). Unlike the identification of substrates for Akt, where many physiological substrates and phosphorylation sites have been discovered because of a specific consensus sequence for Akt phosphorylation (38), identification of substrates for PKC-ζ has been difficult. This is because conventional consensus phosphorylation motifs for protein kinase C ((R/K)*XX*(S/T)*X*(K/R)) (39) have not proven robust or specific for all protein kinase C isoenzymes (40). For example, in IKKβ, the amino acid sequence surrounding PKC-ζ phosphorylation sites at Ser^{177} and Ser^{181} (11, 41) does not conform to either the general protein kinase C consensus motif (39) or an optimal PKC-ζ consensus motif based upon screening of a peptide library (40).

IRS-1 May Be a Novel Physiological Substrate for PKC-ζ—IRS-1 is a substrate of the insulin receptor tyrosine kinase that plays an important role in coupling signaling by the insulin receptor with activation of multiple downstream pathways, including PI 3-kinase and SHP-2 (4, 12). Increased serine phosphorylation of IRS-1 has been implicated in impairment of IRS-1 tyrosine phosphorylation and may be one mechanism underlying acquired insulin resistance (16, 17, 42). Feedback control of IRS-1 may exist because Ser/Thr kinases down-

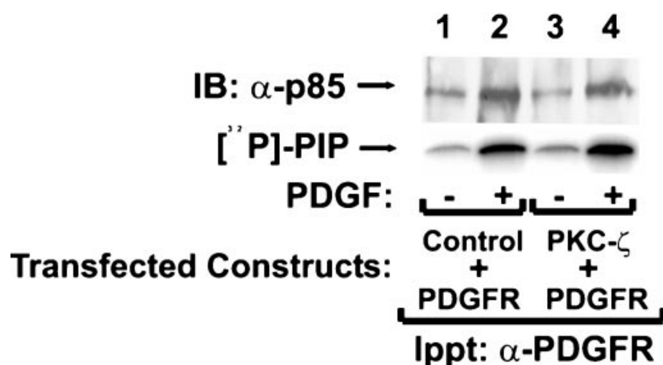

FIG. 7. **Overexpression of PKC-ζ does not inhibit PI 3-kinase activity *per se*.** NIH-3T3^{IR} cells transiently cotransfected with PDGFβ receptor and either a control vector or PKC-ζ were treated without or with PDGF BB (100 ng/ml, 2 min). Cell lysates were immunoprecipitated with anti-PDGFβ receptor antibody followed by immunoblotting with anti-p85 antibody (*top panel*). PI 3-kinase activity associated with the PDGF receptor (*PDGFR*) was assessed in a parallel set of anti-PDGFβ-receptor immunoprecipitates by measuring the ^{32}P-labeled phosphatidylinositol phosphate (PIP) product (*lower panel*). Representative results are shown from experiments that were repeated independently three times. *IB*, immunoblot; *Ippt*, immunoprecipitate.

stream from PI 3-kinase, such as GSK-3 and Akt, are capable of phosphorylating IRS-1 and modulating its function (23–25). When we examined IRS-1 as a potential substrate for PKC-ζ, we found that wild-type PKC-ζ but not kinase-inactive mutant PKC-ζ phosphorylated IRS-1 under *in vitro* conditions, suggesting that IRS-1 is capable of functioning as a direct substrate for PKC-ζ. Moreover, our *in vivo* labeling experiments demonstrating that IRS-1 is a substrate for PKC-ζ in intact cells suggest that IRS-1 is a target of feedback regulation not only by GSK-3 and Akt but also by PKC-ζ. The fact that the interaction between endogenous IRS-1 and PKC-ζ in rat adipose cells was increased upon insulin stimulation is also consistent with an important regulatory role for phosphorylation of IRS-1 by PKC-ζ in insulin signaling pathways.

Functional Consequences of IRS-1 Phosphorylation by PKC-ζ—Because PKC-ζ is downstream from PI 3-kinase, feedback regulation of IRS-1 through phosphorylation by PKC-ζ might be expected to alter the ability of IRS-1 to engage and activate PI 3-kinase. The overexpression of PKC-ζ was sufficient to significantly increase serine phosphorylation of IRS-1 in intact cells. Consistent with a negative feedback role, we observed that the overexpression of PKC-ζ significantly impaired insulin-stimulated tyrosine phosphorylation of IRS-1. These results are in keeping with studies showing that increased serine phosphorylation of IRS-1 secondary to other stimuli is accompanied by impairment in the ability of IRS-1 to undergo tyrosine phosphorylation after insulin stimulation (15–22, 43). In addition, there was some specificity to the impairment of IRS-1 function because the interaction between IRS-1 and SHP-2 appeared to be unaffected by the overexpression of PKC-ζ.

The tyrosine-phosphorylated Y*XX*M motifs on IRS-1 that are predicted to engage and activate PI 3-kinase represent only a fraction of the total number of phosphotyrosine motifs in IRS-1 that couple to downstream signaling molecules (12). Therefore, we specifically examined the consequences of impaired IRS-1 tyrosine phosphorylation on coupling with PI 3-kinase by assessing the coimmunoprecipitation of the p85 regulatory subunit of PI 3-kinase with IRS-1 in the presence or absence of PKC-ζ. Interestingly, the amount of p85 associated with IRS-1 after insulin stimulation was similar in both the presence and absence of PKC-ζ. However, the PI 3-kinase activity associated with IRS-1 was significantly reduced in the presence of PKC-ζ after both 2 and 60 min of insulin treatment. It is unlikely that the overexpression of PKC-ζ is directly inhibiting PI 3-kinase activity because we did not observe any significant ability of PKC-ζ to impair PDGF-stimulated PI 3-kinase activity in anti-PDGF receptor immunoprecipitates. In a previous study, we showed that a mutant human IRS-1 with substitutions of Phe for Tyr at positions 465, 612, 632, 662, 941, and 987 did not bind and activate PI 3-kinase and was incapable of mediating translocation of GLUT4 in rat adipose cells (33). However, an add-back mutant with Tyr^{612} and Tyr^{632} mimicked the effects of wild-type IRS-1 to activate PI 3-kinase in response to insulin and to mediate translocation of GLUT4 (33). To further define the functional consequences of IRS-1 phosphorylation by PKC-ζ, we examined the effects of the overexpression of PKC-ζ on the ability of IRS1-Y612/Y632 to bind and activate PI 3-kinase. Similar to our results with wild-type IRS-1, the overexpression of PKC-ζ did not impair insulin-stimulated association of p85 with IRS1-Y612/Y632 but was accompanied by a significant decrease in associated PI 3-kinase activity. These results suggest that phosphorylation of IRS-1 by PKC-ζ specifically impairs insulin-stimulated tyrosine phosphorylation at residues 612 and 632 and that this somehow interferes specifically with the ability to activate PI 3-kinase. It is likely that phosphorylation of additional tyrosine residues is also affected because total IRS-1 tyrosine phosphorylation in response to insulin was substantially decreased by the overexpression of PKC-ζ. Nevertheless, the functional impairment of IRS-1 caused by the overexpression of PKC-ζ did not appear to interfere with insulin-stimulated interactions between IRS-1 and SHP-2.

It is somewhat surprising that the decrease in insulin-stimulated tyrosine phosphorylation of IRS-1 caused by the overexpression of PKC-ζ was accompanied by a decrease in IRS-1-associated PI 3-kinase activity without a change in p85 binding. In some studies, serine phosphorylation of IRS-1 causes both decreased association of p85 with IRS-1 and decreased PI 3-kinase activity (14, 22, 25). However, many studies examining the effects of IRS-1 serine phosphorylation report either p85 association with IRS-1 or IRS-1-associated PI 3-kinase activity alone but do not correlate p85 binding with PI 3-kinase activity (15, 18, 21, 43). When Y*XX*M motifs in IRS-1 are tyrosine-phosphorylated in response to insulin, the SH2 domains of p85 bind to these sites on IRS-1, resulting in activation of the preassociated p110 catalytic subunit of PI 3-kinase (12, 44). Therefore, one might expect p85 binding to correlate with PI 3-kinase activity. However, the tandem SH2 domains of p85 must be occupied simultaneously for full activation of PI 3-kinase (44–47). It is possible that Ser/Thr phosphorylation of IRS-1 by PKC-ζ may affect the geometry of interactions between tandem SH2 domains in p85 and Y*XX*M motifs in IRS-1 such that differences in coimmunoprecipitation of p85 with IRS-1 are undetectable but full activation of PI 3-kinase activity is impaired.

Feedback regulation of IRS-1 function by downstream Ser/Thr kinases may be an important general property of insulin signal transduction. Direct phosphorylation of IRS-1 by GSK-3 negatively modulates IRS-1 function (24) whereas phosphorylation of IRS-1 by Akt may have both positive and negative effects on signaling by IRS-1 (23, 25). Paz *et al.* (23) suggest that Akt phosphorylation of IRS-1 on four serine residues in the PTB domain of IRS-1 enhances IRS-1 tyrosine phosphorylation in response to insulin. On the other hand, Li *et al.* (25) have implicated Akt pathways in negative modulation of IRS-1 function involving serines 632, 662, and 731. It will be of great interest to identify specific PKC-ζ phosphorylation sites on IRS-1 in future studies.

Conclusions—We have identified IRS-1 as a potential phys-

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iological substrate of PKC-ζ. Phosphorylation of IRS-1 by PKC-ζ represents an additional example of negative feedback regulation of IRS-1 by a Ser/Thr kinase downstream from PI 3-kinase. The existence of multiple feedback regulatory mechanisms at the level of IRS-1 emphasizes the critical importance of IRS-1 in insulin signaling and may have implications for understanding the pathophysiology of insulin resistance in diseases such as diabetes and obesity.

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Protein Kinase C-ζ Phosphorylates Insulin Receptor Substrate-1 and Impairs Its Ability to Activate Phosphatidylinositol 3-Kinase in Response to Insulin

Lingamanaidu V. Ravichandran, Diana L. Esposito, Judy Chen and Michael J. Quon

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