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Protein Kinase C-α Regulates Insulin Action and Degradation by Interacting with Insulin Receptor Substrate-1 and 14-3-3ε*

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Francesco Oriente[‡], Francesco Andreozzi[§], Chiara Romano[‡], Giuseppe Perruolo[‡], Anna Perfetti[‡], Francesca Fiory[‡], Claudia Miele[‡], Francesco Beguinot[‡], and Pietro Formisano[‡1]

From the [‡]Dipartimento di Biologia e Patologia Cellulare e Molecolare and Istituto di Endocrinologia ed Oncologia Sperimentale del CNR, Federico II University of Naples, Via Pansini 5, 80131 Naples and the [§]Dipartimento di Medicina Sperimentale e Clinica, Magna Graecia University of Catanzaro, Via Campanella 115, 88100 Catanzaro, Italy

Protein kinase C (PKC)-α exerts a regulatory function on insulin action. We showed by overlay blot that PKCα directly binds a 180-kDa protein, corresponding to IRS-1, and a 30-kDa molecular species, identified as 14-3-3ε. In intact NIH-3T3 cells overexpressing insulin receptors (3T3-hIR), insulin selectively increased PKCα co-precipitation with IRS-1, but not with IRS-2, and with 14-3-3ε, but not with other 14-3-3 isoforms. Overexpression of 14-3-3ε in 3T3-hIR cells significantly reduced IRS-1-bound PKCα activity, without altering IRS-1/PKCα co-precipitation. 14-3-3ε overexpression also increased insulin-stimulated insulin receptor and IRS-1 tyrosine phosphorylation, followed by increased activation of Raf1, ERK1/2, and Akt/protein kinase B. Insulin-induced glycogen synthase activity and thymidine incorporation were also augmented. Consistently, selective depletion of 14-3-3ε by antisense oligonucleotides caused a 3-fold increase of IRS-1-bound PKCα activity and a similarly sized reduction of insulin receptor and IRS-1 tyrosine phosphorylation and signaling. In turn, selective inhibition of PKCα expression by antisense oligonucleotides reverted the negative effect of 14-3-3ε depletion on insulin signaling. Moreover, PKCα inhibition was accompanied by a >2-fold decrease of insulin degradation. Similar results were also obtained by overexpressing 14-3-3ε. Thus, in NIH-3T3 cells, insulin induces the formation of multimolecular complexes, including IRS-1, PKCα, and 14-3-3ε. The presence of 14-3-3ε in the complex is not necessary for IRS-1/PKCα interaction but modulates PKCα activity, thereby regulating insulin signaling and degradation.

Insulin action on target tissues requires insulin receptor (IR)[2] tyrosine autophosphorylation and activation of the intrinsic tyrosine kinase (1). Phosphorylated insulin receptor substrates (IRSs) then serve as adaptor proteins for a number of signal transduction molecules (2, 3). Therefore, multimolecular complexes, including IRSs, constitute a major platform through which insulin signals are generated. Indeed, it has been demonstrated that the protein/protein interaction events engaged by the IRSs are necessary for insulin induction of specific enzymatic activities and/or for the targeting of signaling molecules to the proper intracellular location (2, 3).

Besides the pivotal function in transducing insulin biologic effects, IRS proteins may also serve as negative regulators of insulin signals. Several *bona fide* inhibitors of insulin action, such as tyrosine phosphatases and serine/threonine kinases, have been shown to interact with IRSs (3–5). Both classes of molecules are involved in turning off insulin signals. Tyrosine phosphatases may remove the phosphorylation of key tyrosines both on the IR and on intracellular substrates (4–6), thereby inhibiting their signaling functions. Also, phosphorylation on serine/threonine residues often interferes with tyrosine phosphorylation (7, 8).

Among serine/threonine kinases, a crucial role in inhibiting insulin signals is played by the protein kinase C (PKC) family members. Several PKC isoforms have been implicated as inhibitors of insulin signal transduction (8–12). Overexpression of PKCα, for instance, has been shown to inhibit insulin signaling in cultured cell systems (9, 10, 12). PKCα is activated by agents known to interfere with insulin action, such as angiotensin II (13), endothelin-1 (14, 15), advanced glycation end products (16), and by prolonged exposure to high glucose concentrations (17). Murine models of PKCα gene ablation also exhibit increased sensitivity to insulin, further supporting the concept of the PKCα negative role in insulin signaling (18).

PKCs are present in multimolecular complexes inside the cells (19, 20), and their activity is finely tuned by changes in the extracellular and/or intracellular milieu (21–23). However, the mechanisms by which PKCα impinges on insulin action remain poorly defined. It is known that it may phosphorylate IR (9, 11), IRS-1 (10), as well as other receptor and nonreceptor tyrosine kinases (24–26) on serine/threonine residues. These phosphorylations may block further tyrosine phosphorylation or induce intracellular degradation of the targeted proteins (9–12, 24–26).

In this work, we have shown that PKCα directly binds insulin-activated IRS-1 and provided evidence that adaptors of the 14-3-3 family participate in the complex formation and down-regulate PKCα activity. This, in turn, removes the inhibitory constraint exerted by PKCα, enhancing IR tyrosine kinase activity and signaling. In addition, we have shown that 14-3-3 modulation of PKCα also controls insulin intracellular sorting toward degradation.

EXPERIMENTAL PROCEDURES

Materials—Media, sera, antibiotics for cell culture, the Lipofectamine reagent, and antibodies toward PKCδ were from Invitrogen. Recombinant PKCα was from Calbiochem-Novabiochem. Insulin receptor α-subunit and IRS-1 and IRS-2 antibodies were purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). Phosphotyrosine antibody was purchased from Transduction Laboratories. PKCα, phos-

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[1] To whom correspondence should be addressed: Dept. di Biologia e Patologia Cellulare e Molecolare, Università di Napoli "Federico II," Via S. Pansini 5, 80131 Naples, Italy. Tel.: 39-081-7463608; Fax: 39-081-7463235; E-mail: fpietro@unina.it.

[2] The abbreviations used are: IR, insulin receptor; PKC, protein kinase C; IRS, insulin receptor substrate; PKB, protein kinase B; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; h, human; DMEM, Dulbecco's modified Eagle's medium; BSA, bovine serum albumin.

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pho-PKCα, and 14-3-3-specific antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Raf, phospho-Raf, MAPK, phospho-MAPK, Akt1/2, phospho-Ser-Akt1/2, Foxo1, and phospho-Ser-Foxo1 were obtained from New England Biolabs (Beverly, MA). pCDNA3-14-3-3ε construct was a generous gift of Dr. G. Viglietto (University of Magna Graecia, Catanzaro, Italy). Phosphorothioate 14-3-3ε (ASε), PKCα (AS-PKCα), and PKCδ (AS-PKCδ) oligodeoxynucleotides antisense were generated with the following sequences: 5′-CTCTCGCTCTTCCAT-3′, 5′-CAGCCATGGTTCCCCCCAAC-3′, and 5′-AGGGTGCCATGAT-GGA-3′, respectively (PRIM, Milan, Italy). For control, scrambled oligodeoxynucleotides based on 14-3-3ε (Sε) (5′-TCCTCTCGCTCTACT-3′), PKCα (S-PKCα) (5′-CCAGTCACTCGCACCATCGC-3′), and PKCδ (S-PKCδ) (5′-TCGATCATGGCACCCT-3′) sequences were also obtained. Protein electrophoresis reagents were purchased from Bio-Rad, and Western blotting, ECL reagent, and ^{125}I-insulin were from Amersham Biosciences. All other chemicals were from Sigma.

Cell Culture and Transfection—NIH-3T3 fibroblasts overexpressing human insulin receptor have been described previously (27). Cells were cultured at 37 °C in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 2% L-glutamine, 10,000 units/ml penicillin, 10,000 μg/ml streptomycin. Transient transfection of the phosphorothioate oligonucleotides and of the 14-3-3ε plasmid was performed by the Lipofectamine method according to the manufacturer's instructions. For these studies, 60–80% confluent cells were washed twice with Opti-MEM (Invitrogen) and incubated for 8 h with 5 μg of plasmid construct or with 12 μg of oligonucleotides and 45–60 μl of the Lipofectamine reagent. The medium was then replaced with DMEM with 10% fetal calf serum, and the cells were further incubated for 15 h before being assayed.

Overlay Blot Assay—Protein lysates were obtained by cells stimulated with insulin for different times as described previously (28). Equal amounts (500 μg) of proteins were immunoprecipitated with anti-IR or IRS-1 antibodies, separated by 7 or 12% SDS-PAGE, and transferred on 0.45-μm Immobilon-P membranes (Millipore, Bedford, MA). The filters were incubated 2 h at room temperature in 5% (w/v) nonfat dry milk/NaCl/Tris (20 mM Tris, 140 mM NaCl, pH 7.6), 0.1% Tween 20 (blocking solution). At this time, recombinant biotinylated PKCα was supplemented with the lipid activators (10 mM phorbol 12-myristate 13-acetate, 0.28 mg/ml phosphatidylserine, and 4 mg/ml dioleine, final concentrations). The phosphorylation solution (20 μM ATP, 1 mM $CaCl_2$, 20 mM $MgCl_2$, and 4 mM Tris, pH 7.5, final concentrations) was then added to those mixtures. The reaction mixtures were further incubated for 10 min at room temperature, rapidly cooled on ice, and added to the blocking solution. After 1 h of incubation at room temperature, the blots were washed twice with NaCl, Tris, 0.1% Tween 20 and then incubated with 0.5% formaldehyde for 10 min. The filters were washed three times with 2% glycine and once with NaCl, Tris, 0.1% Tween 20. PKCα-bound proteins were detected by horseradish peroxidase-bound streptavidin blotting and autoradiography.

Western Blot Analysis and Immunoprecipitation Procedure—For these studies, cells were solubilized in lysis buffer (50 mM Hepes, pH 7.5, 150 mM NaCl, 10 mM EDTA, 10 mM $Na_4P_2O_7$, 2 mM Na_3VO_4, 100 mM NaF, 10% glycerol, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin) for 4 h at 4 °C. Cell lysates were clarified by centrifugation at 5,000 × *g* for 20 min, separated by SDS-PAGE, and transferred on 0.45-μm Immobilon-P membranes. Upon incubation with primary and secondary antibodies, immunoreactive bands were detected by ECL according to the manufacturer's instructions. Immunoprecipitation assays were accomplished as described previously (29).

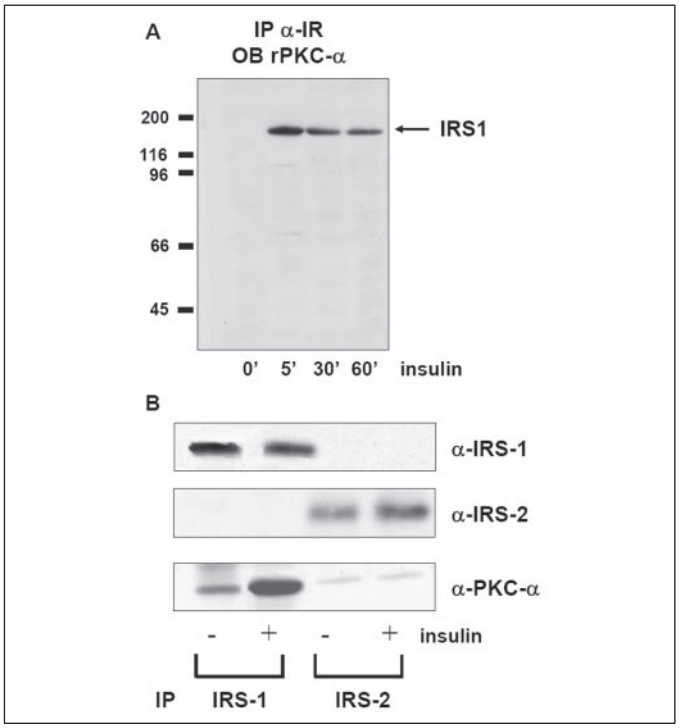

FIGURE 1. **PKCα interaction with IRS proteins.** *A*, 3T3-hIR cells were stimulated with 100 nM insulin for the indicated times, and the cellular extracts (500 μg) were immunoprecipitated (*IP*) with anti-IR antibodies and loaded on SDS-PAGE. Gels were then transferred on nitrocellulose filters and incubated with recombinant biotinylated PKCα (*OB rPKCα*) as described under "Experimental Procedures." *B*, lysates (300 μg), obtained from 3T3-hIR cells treated or not with 100 nM insulin for 30 min were immunoprecipitated with IRS-1 or IRS-2 antibodies followed by blotting with IRS-1, IRS-2, or PKCα antibodies, ECL, and autoradiography. The autoradiographs shown are representative of three (*A*) and four (*B*) independent experiments.

PKCα Activity Assay—PKCα activity was assayed as reported in Formisano *et al.* (see Ref. 30 and modified as indicated in Ref. 29). Briefly, for these assays the cells were solubilized in 20 mM Tris, pH 7.5, 0.5 mM EDTA, 0.5 mM EGTA, 25 μg/ml aprotinin, and 25 μg/ml leupeptin (extraction buffer). Supernatants were further centrifuged at 60,000 × *g* for 2 h, and pellets were solubilized with extraction buffer supplemented with 0.5% Triton X-100. Soluble pellets were immunoprecipitated for 18 h with PKCα or IRS-1 antibody and incubated with protein A-Sepharose for 2 h. Immunocomplexes were supplemented with the lipid activators (0.32 mg/ml phosphatidylserine and 0.032 mg/ml diacylglycerol, final concentrations), and the phosphorylation reaction was initiated by adding the substrate solution (50 mM PKCα substrate peptide, 0.5 mM ATP, 0.25 mM EGTA, 0.4 mM $CaCl_2$, 0.1 mg/ml bovine serum albumin, 20 mM Tris, pH 7.5, 10 mM $MgCl_2$, and 10 μCi/ml (3000 Ci/mmol) [γ-^{32}P]ATP, final concentrations). The reaction mixture was further incubated for 30 min at room temperature, and the phosphorylation reaction was terminated by adding 7.5 M guanidine hydrochloride and spotting on phosphocellulose discs. Disc-bound radioactivity was quantitated by liquid scintillation counting.

Determination of Thymidine Incorporation and of Glycogen Synthase Activity—Twenty four-well plates were seeded with 10^6 cells/plate in 1 ml of DMEM supplemented with 10% fetal calf serum. After the transient transfection with plasmid construct or specific antisense oligonucleotides, the medium was replaced with DMEM with 10% fetal calf serum for 24 h. Then the cells were starved with DMEM containing 0.25% BSA and no serum at 37 °C. After an additional 24 h, the medium was removed again and replaced with DMEM, 0.25% BSA with or without 100 nM insulin. Incubation was prolonged for an additional 16 h, and

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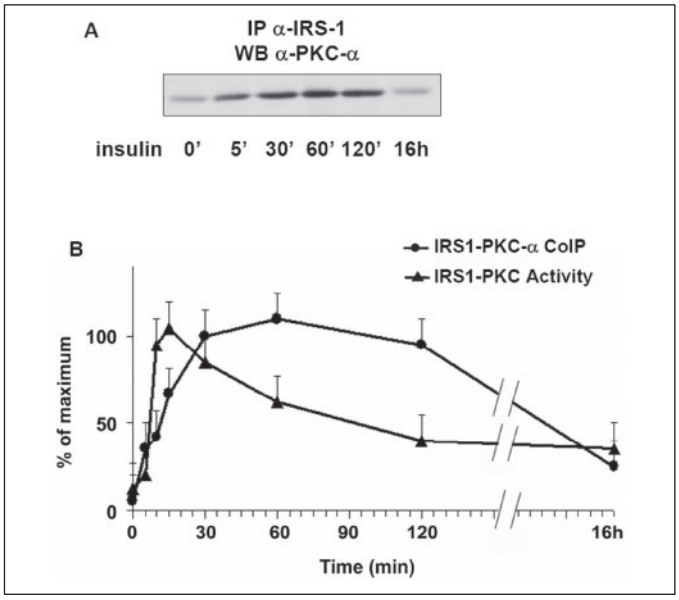

FIGURE 2. **Time course of IRS-1 co-precipitation with PKCα.** *A*, co-precipitation between IRS-1 and PKCα was evaluated in 3T3-hIR cellular extracts immunoprecipitated (*IP*) with IRS-1 antibodies after 100 nM insulin stimulation for the indicated times. Samples were either immunoblotted (*IB*) with PKCα antibodies (*A* and *B*) or assayed for PKCα activity (*B*) as described under "Experimental Procedures." *A*, the autoradiograph is representative of four independent experiments. *B*, the *line graph* represents the mean ± S.D. of duplicate determinations in four independent experiments (for PKCα activity) and the mean ± S.D. of the densitometric analysis for IRS-1/PKCα co-precipitation experiments.

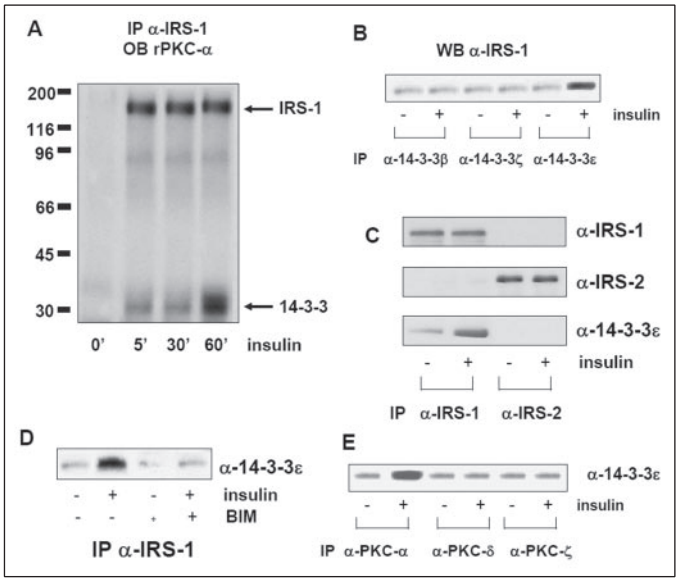

FIGURE 3. **Insulin induces the formation of complexes including IRS-1, PKCα, and 14-3-3ε.** *A*, 3T3-hIR cells were stimulated with 100 nM insulin for the indicated times. The cellular extracts (500 μg) were immunoprecipitated (*IP*) with IRS-1 antibodies and analyzed for PKCα overlay assays as indicated under "Experimental Procedures" and in the legend to Fig. 1. *B*, lysates (300 μg) from cells exposed to 100 nM insulin for 30 min were immunoprecipitated with 14-3-3β, -ε, and -ζ antibodies and blotted with IRS-1 antibodies. *WB*, Western blot. *C*, co-precipitation between IRS-1 or IRS-2 and 14-3-3ε was evaluated in 3T3-hIR cellular extracts before and after 100 nM insulin stimulation for 30 min. The same filter was subsequently re-probed with IRS-1or IRS-2 antibodies. *D*, cells were treated or not treated with 100 nM bisindolylmaleimide (*BIM*) before insulin stimulation (100 nM insulin for 30 min). Cell extracts were then precipitated with IRS-1 antibodies and immunoblotted with 14-3-3ε antibodies. *E*, 3T3-hIR cells lysates obtained as described previously were immunoprecipitated with antibodies against different isoforms of PKC (α, δ, and ζ) and blotted with 14-3-3ε antibodies. The autoradiographs shown are representative of at least three independent experiments.

incubation media were replaced with the same media supplemented with [^{3}H]thymidine (500 nCi/ml). 4 h later, the cells were washed with ice-cold 0.9% NaCl and then with ice-cold 20% trichloroacetic acid followed by solubilization with 1 N NaOH. Radioactivity was quantitated by liquid scintillation counting. Glycogen synthase activity was assayed in cell extracts as described by Miele *et al.* (16).

Insulin Internalization and Degradation—Cells were washed three times with ice-cold KRP solution (120 mM NaCl, 5 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 100 mM Na_2HPO_4), pH 7.8, and incubated at 4 °C for 16 h with 30 pM ^{125}I-insulin diluted in binding buffer (KRP, pH 7.8, containing 2% BSA and 1.3 mM $CaCl_2$). Insulin internalization and degradation were measured as described previously (29). Briefly, cells were rinsed with KRP, pH 7.8, in order to remove the unbound ligand and further incubated at 37 °C in binding buffer. After ice-cold acid washes, cellular radioactivity was considered internalized insulin. Alternatively, cells were re-incubated at 37 °C, and aliquots of the incubation medium were removed to determine the amount of ^{125}I-insulin released from the cells. Degraded insulin was assessed by determining the trichloroacetic acid solubility of ^{125}I-insulin products.

RESULTS

PKCα Interaction with IRS-1 in 3T3-hIR Cells—NIH-3T3 fibroblasts expressing human wild-type insulin receptors (3T3-hIR cells) were treated with 100 nM insulin for 5, 30, and 60 min. Cell lysates were precipitated with insulin receptor antibodies and tested in overlay assays for their ability to bind recombinant biotinylated PKCα. A major 180-kDa band was revealed upon insulin stimulation of the cells (Fig. 1*A*). Both the apparent molecular weight and mass spectrometric analysis indicated that the 180-kDa species corresponded to IRS-1. The observation that the maximal interaction occurred 5 min after insulin exposure was also consistent with the timing of the IR interaction with IRS proteins. To verify the possibility that IRSs could interact with PKCα in intact cells, we performed selective co-precipitation experiments. PKCα was detectable in IRS-1 and, at very low levels, in IRS-2 precipitates (Fig. 1*B*). Moreover, the exposure of 3T3-hIR cells to insulin increased PKCα/IRS-1 co-precipitation in a time-dependent manner (Fig. 2*A*). It was enhanced by 2.5-fold in 5 min, rising to 4-fold in 1 h, and then returning to basal levels after 16 h of insulin treatment. PKCα activity was then measured in IRS-1 precipitates. Again, phosphorylation of the specific PKC substrate peptide was dependent on the length of insulin exposure. It featured a rapid increase that peaked at 15 min followed by a slower decrease (Fig. 2*B*).

14-3-3ε Interaction with PKCα and IRS-1 in 3T3-hIR Cells—We hypothesized that the apparent discrepancy between the relative amounts of IRS-1-precipitated PKCα activity and PKCα protein (Fig. 2*B*) could be due, at least in part, to the presence of PKC modulators within the IRS-1 complexes.

In order to analyze this possibility, IRS-1 precipitates were probed with recombinant PKCα in overlay assays (Fig. 3*A*). The expected 180-kDa IRS-1 band was observed, and an additional intensely stained 30-kDa band was detectable in IRS-1 precipitates obtained from insulin-stimulated cells. No specific band was detected in IRS-2 precipitates (data not shown). Microsequencing of the isolated 30-kDa species revealed an amino acid composition consistent with that of the adaptor proteins of the 14-3-3 family.

To investigate whether a specific 14-3-3 isoform could interact with IRS-1, cell lysates from insulin-stimulated and unstimulated cells were precipitated with selective antibodies and blotted with IRS-1 antibodies (Fig. 3*B*). We used 14-3-3β, -ζ, and -ε antibodies because prior immunoblot experiments of whole 3T3-hIR cell lysates did not indicate other 14-3-3 isoforms (data not shown). Although a basal signal was detected in all the samples, no change of IRS-1 levels in 14-3-3β and -ζ precipi-

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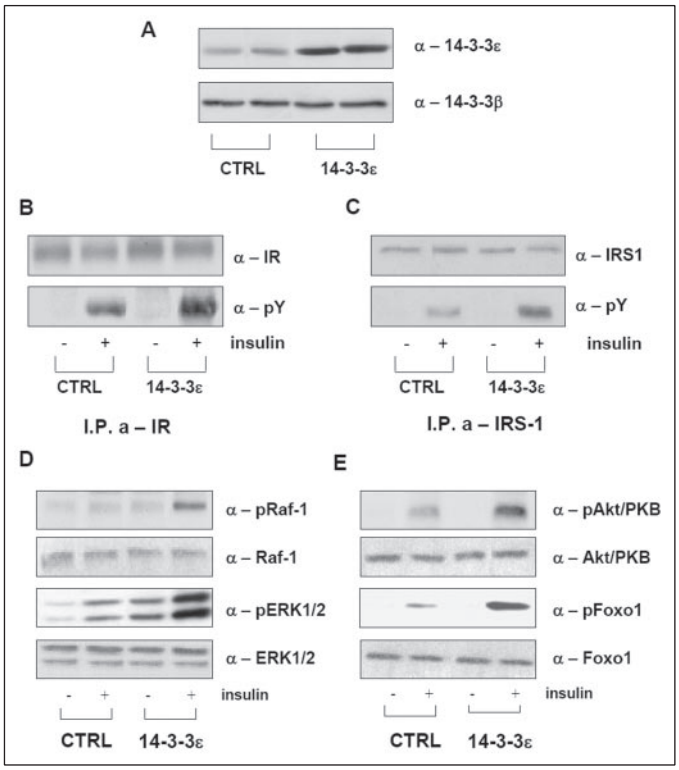

FIGURE 4. **Effect of 14-3-3ε overexpression on IR and IRS-1 tyrosine phosphorylation and signaling.** *A*, 3T3hIR cells were transiently transfected either with a cDNA encoding for the 14-3-3ε isoform or with a control (*CTRL*) vector. Aliquots of the cell lysates were separated by SDS-PAGE and analyzed by Western blot with 14-3-3ε or 14-3-3β antibodies. *B* and *C*, control and 14-3-3ε transfected cells were treated with 100 nM insulin for 5 min and precipitated with IR (*B*) or IRS-1 (*C*) antibodies followed by blotting with IR, IRS-1, and phosphotyrosine antibodies as indicated. The autoradiographs shown are representative of five independent experiments. *IP*, immunoprecipitation. *D* and *E*, 3T3hIR cells were stimulated with 100 nM insulin for 15 min. Lysates were analyzed by immunoblot with the phosphorylated or total form of Raf, ERK1/2, Akt/PKB, and Foxo1 antibodies, as indicated. The autoradiographs shown are representative of five independent experiments.

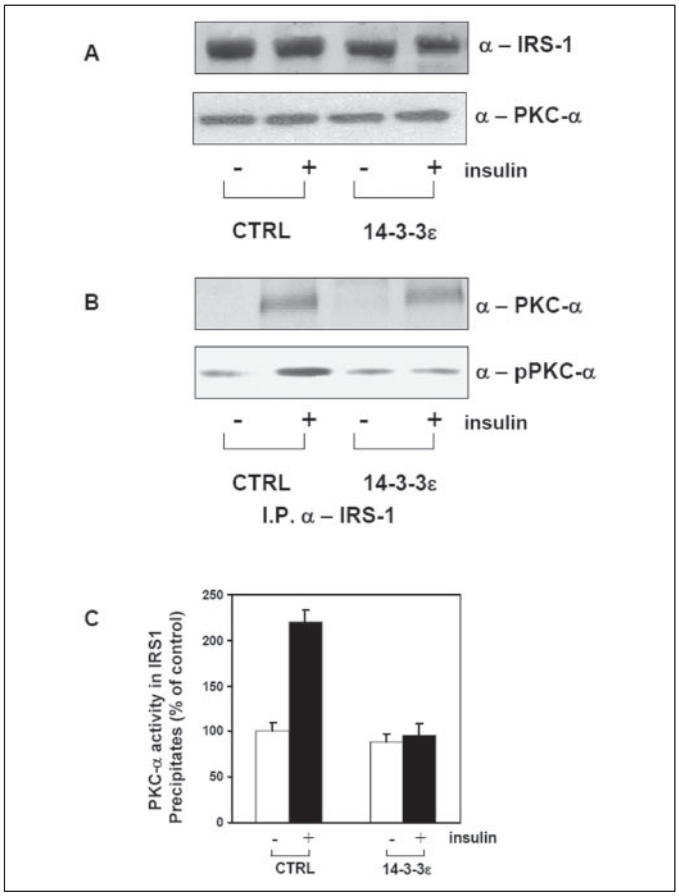

FIGURE 5. **Effect of 14-3-3ε overexpression on IRS-1 interaction with PKCα.** *A*, control (*CTRL*) or 14-3-3ε transfected cells were stimulated with 100 nM insulin for 30 min and the lysates analyzed by immunoblot with IRS-1 and PKCα antibodies. *B*, the same cell extracts were immunoprecipitated (*I.P.*) with IRS-1 followed by blotting with PKCα or Ser[473]-phospho-PKCα antibodies. The autoradiographs shown are representative of at least four independent experiments (*A* and *B*). *C*, 3T3hIR cells untransfected and transfected with 14-3-3ε were stimulated with 100 nM insulin for 30 min. The cells were then lysed and immunoprecipitated with IRS-1 antibodies. PKCα activity was assayed as described under "Experimental Procedures." The *bars* represent the mean ± S.D. of duplicate determinations in four independent experiments.

tates was observed after insulin stimulation. At variance, insulin exposure increased IRS-1 co-precipitation with 14-3-3ε by >2-fold (Fig. 3*B*). Similar results were obtained by precipitating cell lysates with IRS-1 followed by blotting with 14-3-3ε antibodies (Fig. 3*C*). Again, no band corresponding to 14-3-3ε was detected in IRS-2 precipitates both in the presence and in the absence of insulin (Fig. 3*C*). Most interestingly, treatment of the cells with the PKC inhibitor bisindolylmaleimide prevented co-precipitation of 14-3-3ε with IRS-1 (Fig. 3*D*), indicating that PKC activity is necessary for the interaction to occur.

Next, we sought to determine whether 14-3-3ε could bind PKCα in response to insulin in intact cells. As shown in Fig. 3*E*, insulin stimulated 14-3-3ε co-precipitation with PKCα by >3-fold. At variance, no insulin-induced interaction of 14-3-3ε with PKCδ and -ζ was detected (Fig. 3*E*).

Effect of 14-3-3ε Overexpression in Insulin Signaling—We then performed expression studies aimed at verifying whether 14-3-3ε may affect insulin action. By transient transfection of the 14-3-3ε cDNA, the NIH-3T3 cell expression levels of 14-3-3ε were increased by 5-fold above endogenous levels (Fig. 4*A*). In control and in 14-3-3ε-overexpressing cells, insulin increased insulin receptor tyrosine phosphorylation by 3- and 8-fold, respectively, with no difference in basal levels (Fig. 4*B*). In parallel, insulin-stimulated tyrosine phosphorylation of IRS-1 was 2.5-fold higher in the transfected cells compared with the control cells (Fig. 4*C*). Similar increases of insulin-stimulated Raf-1, ERK1/2, Akt/PKB, and Foxo1 phosphorylation were also obtained upon transient transfection of 14-3-3ε (Fig. 4, *D* and *E*).

Overexpression of 14-3-3ε cells did not change the protein levels of IRS-1 and PKCα when assayed in whole cell lysates (Fig. 5*A*). Also, no difference in PKCα levels was detected in IRS-1 precipitates from 14-3-3ε-overexpressing and control cells, whether in the absence or in the presence of insulin (Fig. 5*B*). At variance, although in control cells insulin increased the amount of IRS-1-bound phosphorylated PKCα by >2-fold, no such effect was elicited in the 14-3-3ε-overexpressing cells (Fig. 5*B*). In parallel with phosphorylation, insulin exposure caused a 2-fold increase of PKC activity in IRS-1 precipitates in the 3T3-hIR cells but not in 14-3-3ε-overexpressing cells (Fig. 5*C*). Therefore, it appears that 14-3-3ε plays a role in inhibiting IRS-1-bound PKCα activity and in increasing IR/IRS-1 tyrosine phosphorylation and downstream signaling.

Silencing of 14-3-3ε in 3T3-hIR Cells—To address the functional significance of the endogenous 14-3-3ε protein in insulin signaling, we used a specific phosphorothioate antisense oligonucleotide (ASε) to block 14-3-3ε expression. The ASε caused a >70% reduction of 14-3-3ε expression compared with a scrambled oligonucleotide (SCε) with no effect on the 14-3-3β and -ζ isoforms (Fig. 6*A*). Antisense reduction of 14-3-3ε levels was accompanied by a constitutive increase of IRS-1 co-

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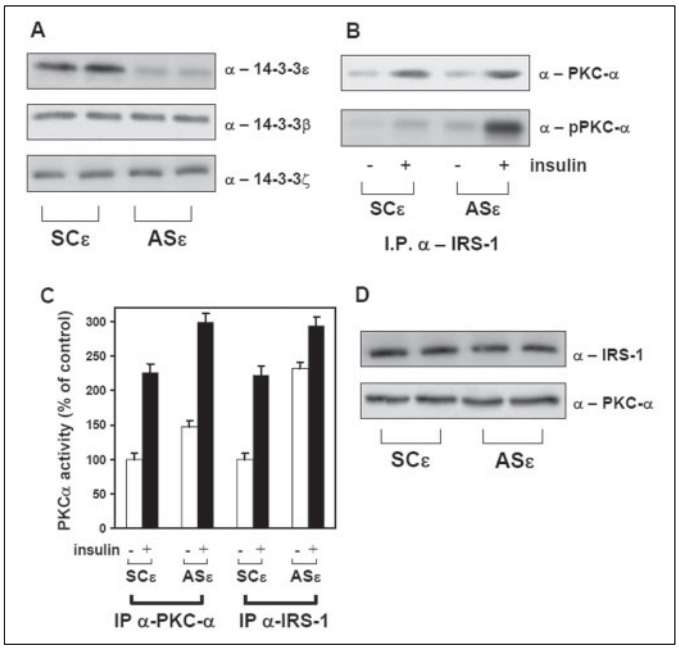

FIGURE 6. **Effect of 14-3-3ε inhibition on IRS-1 interaction with PKCα.** *A*, 3T3hIR cells were transiently transfected with specific 14-3-3ε phosphorothioate antisense oligonucleotides (*ASε*) or a scrambled oligonucleotide (*SCε*). Aliquots of the cell lysates were separated by SDS-PAGE and analyzed by Western blot with 14-3-3ε, -β, and -ζ antibodies. *B*, cells transfected with ASε or SCε were treated with 100 nM insulin and precipitated with IRS-1 antibodies followed by blotting with PKCα or Ser[473]-phospho-PKCα antibodies. *C*, alternatively, PKCα activity was assayed in PKCα and in IRS-1 precipitates, as described under "Experimental Procedures." The *bars* represent the mean ± S.D. of duplicate determinations in four independent experiments. *IP*, immunoprecipitation. *D*, lysates obtained as previously described (*A*) were blotted with IRS-1 or PKCα antibodies as indicated. The autoradiographs shown are representative of at least four independent experiments (*A*, *B*, and *D*).

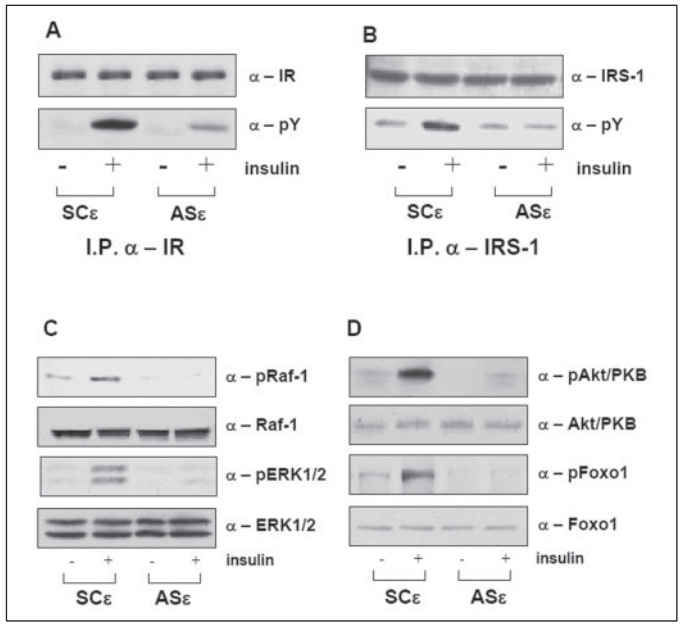

FIGURE 7. **Effect of 14-3-3ε inhibition on IR and IRS-1 tyrosine phosphorylation and on Akt/PKB and MAPK signaling.** 3T3hIR cells were transiently transfected with a specific 14-3-3ε phosphorothioate antisense oligonucleotide (*ASε*) or a scrambled oligonucleotide (*SCε*). Cell were treated with 100 nM insulin for 5 min (*A* and *B*) or for 15 min (*C* and *D*). *A*, aliquots of the cell lysates were precipitated with IR or IRS-1 antibodies followed by blotting with IR, IRS-1, and phosphotyrosine antibodies (*A* and *B*). Alternatively, lysates were blotted with the phosphorylated or the total form of Raf, ERK1/2, Akt/PKB, and Foxo1 antibodies as indicated (*C* and *D*). The autoradiographs shown are representative of at least four independent experiments. *I.P.*, immunoprecipitation.

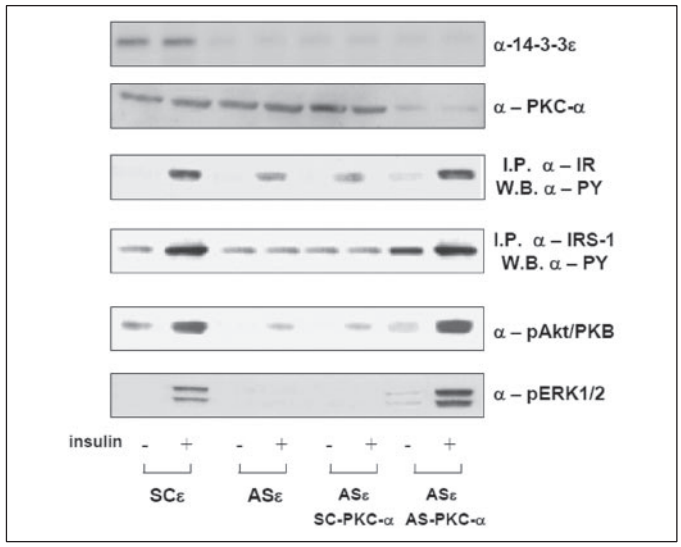

FIGURE 8. **Effect of simultaneous 14-3-3ε and PKCα inhibition on IR and IRS-1 tyrosine phosphorylation and on Akt/PKB and MAPK.** 3T3hIR cells were transiently transfected with a 14-3-3ε (*ASε*) and/or with a specific PKCα phosphorothioate antisense oligonucleotide (*AS-PKCα*) or scrambled oligonucleotide (*SC-PKCα*). Cells were treated with 100 nM insulin for 5 min, and cell lysates were precipitated with IR or IRS-1 antibodies followed by blotting with phosphotyrosine antibodies. Alternatively, lysates were blotted with 14-3-3ε, PKCα, or with the phosphorylated forms of Akt/PKB and ERK1/2 antibodies. The autoradiographs shown are representative of at least three independent experiments. *I.P.*, immunoprecipitation; *W.B.*, Western blot.

precipitation with the phosphorylated form of PKCα, which was further augmented upon insulin exposure of the cells (Fig. 6*B*). In parallel, total and IRS-1-associated PKCα activities were constitutively increased by 1.5- and 2.5-fold, respectively, and further enhanced by insulin (Fig. 6*C*). At variance, PKCα levels in IRS-1 precipitates were almost identical in SCε- and in ASε-treated cells, in basal conditions, and were stimulated by insulin to a similar extent (Fig. 6*B*). Also, PKCα immunodetection in whole cell lysates was not changed by the treatment with both oligonucleotides (Fig. 6*D*).

Consistent with a negative role of 14-3-3ε depletion in insulin signaling, IR- and IRS-1-tyrosine phosphorylations were decreased by 70 and 90%, respectively, in cells treated with the ASε, compared with the control cells (Fig. 7, *A* and *B*). Raf1, ERK1/2, Akt/PKB, and Foxo1 phosphorylations were also drastically reduced by the inhibition of 14-3-3ε expression (Fig. 7, *C* and *D*).

IR, IRS-1, ERK1/2, and Akt/PKB phosphorylations were also analyzed in the presence of a specific PKCα phosphorothioate antisense oligonucleotide (AS-PKCα), which reduced PKCα expression by about 70%. Most interestingly, the inhibitory effect exerted by 14-3-3ε silencing was almost completely reverted by the simultaneous PKCα depletion (Fig. 8). Expression of IR, IRS-1, ERK1/2, and Akt/PKB was unchanged, however (data not shown).

Modulation of Insulin Action by 14-3-3ε—We next assessed whether insulin biological effects were modulated by 14-3-3ε. To this end, thymidine incorporation into DNA and glycogen synthase activity was assayed in 3T3-hIR cells overexpressing 14-3-3ε or in those treated with the ASε (Fig. 9). Consistent with 14-3-3ε effect on early events in insulin signaling, insulin action on both thymidine incorporation (Fig. 9*A*) and glycogen synthase activity (Fig. 9*B*) was enhanced 1.5-fold following 14-3-3ε overexpression and was reduced >80% upon antisense depletion of 14-3-3ε. Again, the effect of 14-3-3ε depletion was reverted when the cells were simultaneously treated with AS-PKCα, although not with a scrambled control (Fig. 9, *A* and *B*).

PKCα and 14-3-3ε Roles in Insulin Degradation—It has been reported previously in 3T3-hIR cells that inhibition of PKC activity

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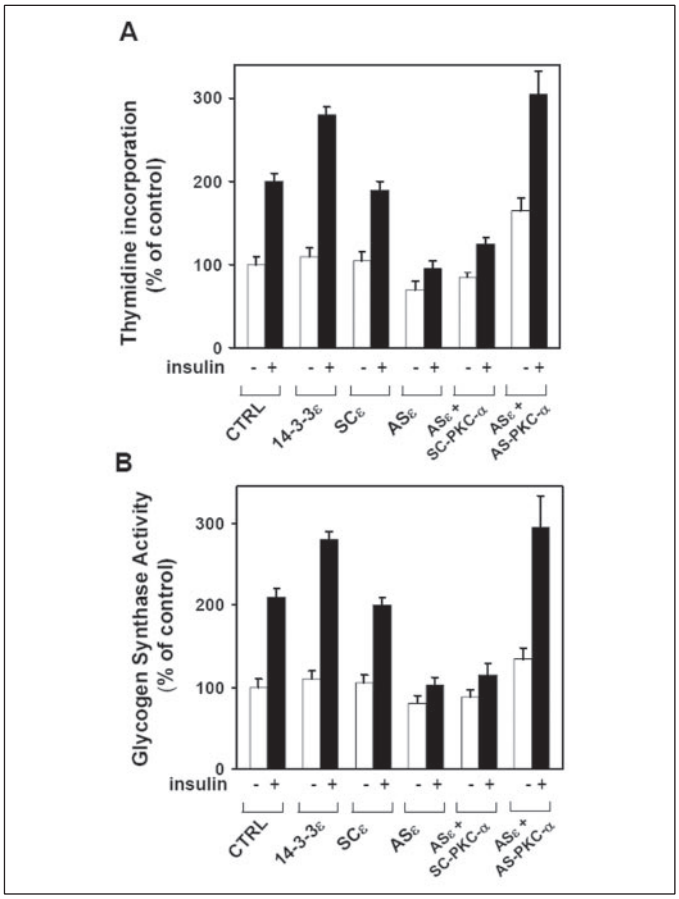

FIGURE 9. **Effect of 14-3-3ε modulation on thymidine incorporation and glycogen synthase activity.** *A*, 6-well plates of untransfected cells or of cells transfected with 14-3-3ε, SCε, or ASε in the presence or absence of scrambled control oligonucleotide (*SC-PKCα*) or PKCα phosphorothioate antisense oligonucleotide (*AS-PKCα*) were incubated or not with 100 nM insulin for 16 h and assayed for [^{3}H]thymidine incorporation as described under "Experimental Procedures." The *bars* represent the mean ± S.D. of triplicate determinations in four independent experiments. *B*, the cells were exposed to 100 nM insulin for 30 min. Glycogen synthase activity was then assayed as in Ref. 16. The *bars* represent the mean ± S.D. of triplicate determinations in four independent experiments. *CTRL*, control.

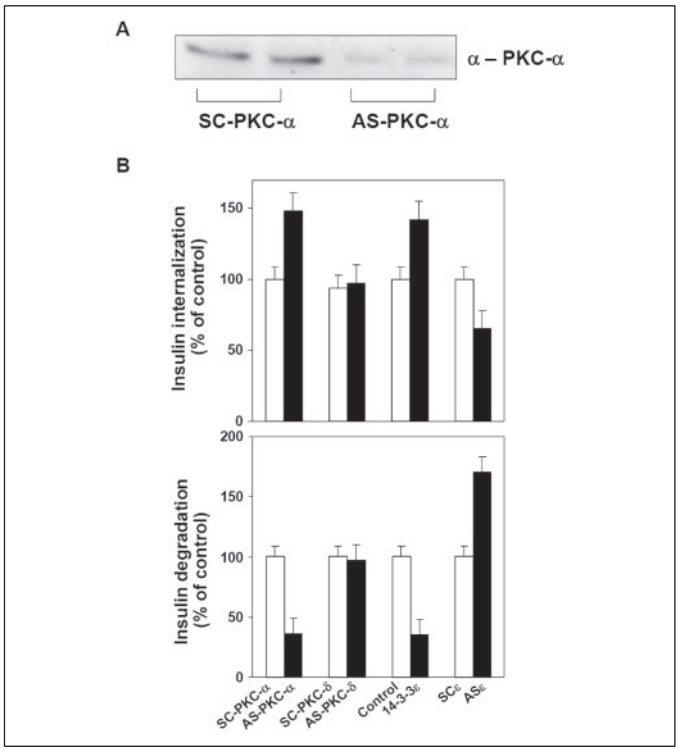

FIGURE 10. **Role of PKCα and 14-3-3ε on insulin internalization and degradation.** *A*, 3T3-hIR cells were transiently transfected with scrambled control oligonucleotide (*SC-PKCα*) or specific PKCα phosphorothioate antisense oligonucleotides (*AS-PKCα*). A Western blot with PKCα antibodies is shown as control. *B*, the same cells were transfected with a PKCα scrambled or antisense oligonucleotides (SC-PKCα and AS-PKCα), PKCδ scrambled or antisense oligonucleotides (SC-PKCδ and AS-PKCδ), a cDNA corresponding to the 14-3-3ε isoform (14-3-3ε), and 14-3-3ε scrambled or antisense oligonucleotides (*SCε* and *ASε*). Insulin internalization and degradation were assessed as indicated under "Experimental Procedures." *B*, the *bars* represent the mean ± S.D. of triplicate determinations in four independent experiments.

reduced the degradation of insulin (27). To investigate further a possible role for PKCα in controlling insulin degradation, 3T3-hIR fibroblasts were transfected with AS-PKCα. (Fig. 10*A*). Selective inhibition of PKCα was accompanied by a 64% decrease of insulin degradation compared with the control untransfected cells or to cells transfected with the scrambled control oligonucleotide (SC-PKCα) (Fig. 10*B*). In contrast, insulin internalization was increased by >50% (Fig 10*B*). Specific PKCδ antisense (AS-PKCδ) and scrambled oligonucleotides (SC-PKCδ) did not change insulin internalization and degradation (Fig. 10, *B* and *C*). Most interestingly, the overexpression of 14-3-3ε also induced a 65% reduction in the insulin degradation, although the ASε block of 14-3-3ε expression caused a 70% increase in the amount of the degraded insulin, as compared with either untransfected or cells transfected with the SCε control oligonucleotide. In parallel with PKCα changes, insulin internalization was increased by about 40% and reduced by about 35%, respectively, in ASε-transfected cells and in those overexpressing 14-3-3ε.

DISCUSSION

Insulin stimulates the activation of PKC isoforms in several tissues and cell types (27, 30–35). It has also been reported that PKC isoforms may form complexes with the IR and phosphorylate several molecules involved in IR-initiated signaling, inhibiting their function (9–12, 27, 36, 37). For instance, PKCα has been reported to inhibit insulin action in both cellular and animal models (17, 18). Here we have used NIH-3T3 fibroblasts expressing human wild-type IR (3T3-hIR cells) and investigated the mechanism of PKCα complex formation with molecules involved in early events of insulin signaling. By overlay blot and co-precipitation experiments, we show that PKCα selectively binds IRS-1 in an insulin-stimulated manner. This finding is consistent with previous reports indicating that IRS-1 expression is required for insulin activation of PKCα in L6 rat skeletal muscle cells (30). Most interestingly, in these same cells, the depletion of IRS-1 also inhibits PKCα co-precipitation with the IR,[3] suggesting the existence of a multimolecular complex, where IRS-1 docks PKCα to the IR. Indeed, overlay blots revealed a direct interaction of PKCα with IRS-1 but not with the IR.

Our experiments also indicated the presence of the scaffold protein 14-3-3ε in IRS-1 precipitates from insulin-stimulated cells. No other 14-3-3 protein, among those expressed in NIH-3T3 cells, co-precipitated with IRS-1 in an insulin-stimulated manner. At variance with these cells, it has been reported that insulin stimulates IRS-1 association with 14-3-3β in 3T3-L1 adipocytes (38). Although abundant in several tissues (39–42), 14-3-3ε is not expressed in the adipocytes. It is possible, therefore, that individual 14-3-3 isoforms play specific roles in different cell types and/or at different stages of differentiation.

14-3-3 proteins often function as molecular adaptors and regulate the

[3] F. Oriente, F. Andreozzi, C. Romano, G. Perruolo, A. Perfetti, F. Fiory, C. Miele, F. Beguinot, and P. Formisano, unpublished results.

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intracellular localization of signaling proteins (43). Mounting evidence also indicates that these proteins modulate the activity of a number of serine/threonine kinases, including members of the PKC family (44–48). In the present report, we show that 14-3-3ε directly interacts with PKCα in the NIH-3T3 cells. Under basal conditions, 14-3-3ε also binds to other PKC isoforms. Insulin, however, selectively induced the co-precipitation of 14-3-3ε with PKCα.

In order to elucidate the role of 14-3-3ε in insulin signaling, we have analyzed the consequences of 14-3-3ε overexpression in the 3T3hIR cells. We found that it significantly reduced IRS-1-bound PKCα activity. Conversely, antisense suppression of 14-3-3ε expression was accompanied by an increase of PKCα activity. These changes in enzymatic activity were not paralleled by changes in the amount of PKCα in IRS-1 precipitates, indicating that 14-3-3ε does not regulate the formation of the IRS-1-PKCα complex. The finding that PKCα independently binds both IRS-1 and 14-3-3ε in overlay assays further supports this hypothesis.

Ogihara *et al.* (49) have shown that the association of 14-3-3 proteins with IRS-1 is increased by the serine phosphatase inhibitor okadaic acid in L6, HepG2, and Chinese hamster ovary cells. We have obtained similar results in 3T3hIR cells (data not shown). In addition, we show that IRS-1 co-precipitation with 14-3-3ε was reduced by the PKC inhibitor bisindolylmaleimide. Thus, one might speculate that, following insulin stimulation, the activated PKCα binds IRS-1, leading to IRS-1 serine/threonine phosphorylation and enabling 14-3-3ε recruitment to the IRS-1-PKCα complex. In the complex, 14-3-3ε down-regulates PKCα activity. Indeed, specific binding sequences for 14-3-3 are potential IRS-1 serine phosphorylation sites (38, 49). Consistent with this hypothesis, we show that, as for the selective inhibition of PKCα (17, 18), 14-3-3ε overexpression is accompanied by improved IR and IRS-1 tyrosine phosphorylation and by increased activation of downstream molecules (*i.e.* Raf-1, ERK1/2, Akt/PKB, and Foxo-1) and of insulin biologic effects. Furthermore, depletion of the endogenous 14-3-3ε reduced insulin-stimulated IR tyrosine phosphorylation and signaling. The negative effect of 14-3-3ε depletion on insulin action is almost completely reverted by the simultaneous inhibition of PKCα expression. Thus, 14-3-3ε may serve as an endogenous regulator of insulin action, by modifying PKCα function in insulin-induced signaling protein complexes.

We have shown previously (27) that pharmacological inhibition of PKC activity reduces insulin degradation. We now provide evidence that PKCα is involved in targeting insulin toward degradation. Indeed, possibly because of the increased receptor autophosphorylation and/or signaling, insulin endocytosis is increased in the cells with reduced PKCα activity (either by antisense depletion or by 14-3-3ε overexpression). The increase of internalization, however, is not followed by a similarly sized hormone degradation, as the selective depletion of PKCα actually reduces the amount of degraded insulin. Moreover, when 14-3-3ε is overexpressed in 3T3hIR cells, the insulin degradation is markedly reduced, paralleling the decrease of PKCα activity. Thus, although IR kinase activation and increased PKCζ signaling may be required for the internalization of the insulin-receptor complex (29, 50), PKCα activation represents a further step necessary for the progression of incoming insulin toward degradation.

In conclusion, in this work we provide the following evidence: (i) insulin stimulates the formation of a multimolecular complex, including IRS-1 and PKCα; (ii) the presence of 14-3-3ε is not necessary for the formation of this complex, but it regulates the activity of PKCα within the complex; and (iii) PKCα inhibits IR/IRS-1 signaling and regulates insulin degradation. This may provide a novel mechanism for the autoregulation of insulin sensitivity and for insulin desensitization in response to a number of PKC-activating agents.

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Protein Kinase C-α Regulates Insulin Action and Degradation by Interacting with Insulin Receptor Substrate-1 and 14-3-3?

Francesco Oriente, Francesco Andreozzi, Chiara Romano, Giuseppe Perruolo, Anna Perfetti, Francesca Fiory, Claudia Miele, Francesco Beguinot and Pietro Formisano

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