The regulatory domain of protein kinase C delta positively regulates insulin receptor signaling

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Abstract

Protein kinase C delta (PKC δ) is induced by insulin to rapidly associate with insulin receptor (IR) and upregulates insulin signaling. We utilized specific JM and CT receptor domains and chimeras of PKC α and PKC δ regulatory and catalytic domains to elucidate which components of PKC δ are responsible for positive regulatory effects of PKC δ on IR signaling. Studies were performed on L6 and L8 skeletal muscle myoblasts and myotubes. PKC δ was preferentially bound to the JM domain of IR, and insulin stimulation increased this binding. Both PKC δ/α and PKC α/δ chimeras (regulatory/catalytic) were bound preferentially to the JM but not to the CT domain of IR. Although IR–PKC δ binding was higher in cells expressing either the PKC δ/α or PKC α/δ chimera than in control cells, upregulation of IR signaling was observed only in PKC δ/α cells. Thus, in response to insulin increases in tyrosine phosphorylation of IR and insulin receptor substrate-1, downstream signaling to protein kinase B and glycogen synthase kinase 3 (GSK3) and glucose uptake were greater in cells overexpressing PKC δ/α and the PKC δ/δ domains than in cells expressing the PKC α/δ domains. Basal binding of Src to PKC δ was higher in both PKC δ/α - and PKC α/δ -expressing cells compared to control. Binding of Src to IR was decreased in PKC α/δ cells but remained elevated in the PKC δ/α cells in response to insulin. Finally, insulin increased Src activity in PKC δ/α -expressing cells but decreased it in PKC α/δ -expressing cells. Thus, the regulatory domain of PKC δ via interaction with Src appears to determine the role of PKC δ as a positive regulator of IR signaling in skeletal muscle.

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Introduction

The protein kinase C (PKC) family of serine–threonine kinases plays important roles in many intracellular signaling events, cell growth, and differentiation (Nishizuka 1988, 1989, 1992). It is generally accepted that the enzymes, when quiescent, are located in the cytoplasm and upon activation are translocated to their sites of action (Einspahr *et al.* 1990). The groups differ from one another in the composition of their regulatory domains.

The insulin receptor (IR) is a heterotetrameric transmembrane glycoprotein composed of two extracellular α -subunits and two transmembrane β -subunits linked by disulfide bonds (White & Kahn 1994). The α -subunits contain the insulin-binding domain, while the transmembrane β -subunits function as a tyrosine-specific protein kinase that undergoes autophosphorylation following insulin binding. This leads to tyrosine phosphorylation of endogenous proteins including the family of insulin receptor substrate (IRS) proteins. Phosphorylated motifs on these proteins serve as binding sites for the recruitment of downstream signaling proteins such as phosphatidylinositol 3-kinase (PI3K) and other protein kinases

(White 1997, 1998, Zick 2004a,b, Biddinger & Kahn 2006). It has been reported that IR interacts with its immediate downstream effectors, IRS and Shc, through distinct receptor regions, and that autophosphorylation of Tyr residues located at the CT domain of the IR can modulate these interactions (Paz *et al.* 2000).

We recently showed that PKC δ plays an important unique role in IR signaling and in the mediation of insulin-induced glucose uptake in skeletal muscle, a major insulin-responsive tissue (Braiman *et al.* 1999a). PKC δ is activated by insulin stimulation upstream of PI3K and is induced by insulin to associate with IR within 1 min (Braiman *et al.* 1999b, 2001b). Moreover, overexpression of PKC δ promotes IR tyrosine phosphorylation and internalization and increases basal glucose uptake; blockade of PKC δ has opposite effects. There is an apparent inconsistency in that while PKC δ is a serine–threonine kinase, and that these kinases downregulate IR signaling, PKC δ has a positive effect in IR regulation, although we also reported that PKC δ has a later effect to serine phosphorylate IR, and may thus also downregulate IR signaling.

In this study, we have attempted to resolve this contradiction by examining the molecular interactions between PKC δ and IR. We studied PKC δ interaction

with individually expressed JM and CT receptor domains. We also utilized chimeras of PKC α and PKC δ regulatory and catalytic domains to elucidate which component of PKC δ may be responsible for effects of PKC δ on IR signaling. Chimeras have been used to delineate the contributions of individual PKC isoforms in a number of systems (see Kronfeld *et al.* 2000, Mandil *et al.* 2001, Blass *et al.* 2002). As insulin stimulation induces PKC δ association with Src tyrosine kinase and increases PKC δ tyrosine phosphorylation, perhaps mediated by the Src family of non-receptor tyrosine kinases (Konishi *et al.* 1997, 2001, Benes & Soltoff 2001, Rosenzweig *et al.* 2004), we examined effects of insulin stimulation on Src and its interactions with IR and PKC δ in the different PKC chimeras. Src was shown to increase insulin-induced tyrosine phosphorylation of IR (Yu *et al.* 1985), and it was reported that Src associates with activated fibroblast growth factor receptor (FGFR) in response to FGF2 and has an important role in the regulation of FGFR1 signaling dynamics (Sandilands *et al.* 2007). In this study, we demonstrate that the regulatory domain of PKC δ is the main determinant of upregulation of IR signaling by PKC δ , most likely via interactions with Src tyrosine kinase.

Materials and methods

Materials

Tissue culture media and serum were purchased from Biological Industries (Beit HaEmek, Israel). Enhanced chemical luminescence (ECL) was performed with antibodies purchased from Bio-Rad and reagents from Sigma. The following antibodies to various proteins were used: anti-PKC antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA and Transduction Laboratories, Lexington, KY, USA); anti-phosphoserine antibodies (Biomedex, Foster City, CA, USA); anti IRS-1 Ser307, anti p-protein kinase B (PKB) Thr308, and anti p-GSK3 Ser9 (Cell Signaling Technology, Beverly, MA, USA); anti-pSrcY529 (Stressgen, Victoria, British Columbia, Canada); HRP-conjugated anti-rabbit and anti-mouse IgG (Bio-Rad). Protease and phosphatase inhibitor cocktails were purchased from Sigma Chemicals. Insulin (Humulin R-recombinant human insulin) was purchased from Lilly France SA (Fergersheim, France).

Cell culture

L8 muscle cells were a gift from Dr D Yaffe (Weizmann Institute of Science, Rehovot, Israel); L6 skeletal muscle cells were obtained from Dr Nava Bashan (Ben-Gurion

University, Beersheba, Israel). L6 cells were grown in alpha modified Eagle's medium supplemented with 10% FCS for 4 days post-confluence, with media changed daily. 3T3 fibroblasts expressing human IR (hIR) or C-terminal (Δ CT) deleted IR (a kind gift from Prof. Hans Haring, University of Tübingen, Tübingen, Germany) were grown in DMEM supplemented with 10% FCS.

Skeletal muscle cultures were prepared from thigh muscles obtained from 1- to 2-day-old neonatal rats as described. The muscles were removed from the limbs, washed in PBS to remove excess blood cells, and then transferred to a Ca²⁺-free, 0.25% trypsin solution containing EDTA (1 mM) for incubation with continuous stirring at 37 °C. Cells were collected after serial trypsinization (successive 15-min periods until all tissues were dispersed) and then centrifuged for 5 min at 500 g. Pellets were resuspended in growth medium and preplated for 20–30 min to reduce the number of fibroblasts. The myoblasts were diluted with growth medium to a concentration of 0.8×10^6 cells/ml for plating in collagen-coated 10-cm plastic tissue culture (10 ml/dish) plates. Cultures were grown in a water-saturated atmosphere of 95% air and 5% CO₂ at 37 °C. On day 4 in culture, myotubes were transferred to starvation medium for 18 h before study.

Preparation of crude cell lysates

Culture dishes (90 mm; Nunc, Thermo Fisher Scientific, Rochester, NY, USA) containing the muscle cells were washed with Ca²⁺/Mg²⁺-free PBS and then mechanically detached in NP-40 buffer containing a cocktail of protease inhibitors and phosphatase inhibitors (Sigma). After scraping, the preparation was centrifuged at 20 000 g for 20 min at 4 °C. The supernatant was used for immunoprecipitation and binding assays.

Western blot analysis

Western blotting was performed as already described (Braiman *et al.* 1999b, Rosenzweig *et al.* 2002, Horovitz-Fried *et al.* 2006a). Briefly, 20–25 μ g protein was electrophoresed through SDS-polyacrylamide gels (7.5 or 10%) and electrophoretically transferred onto Protran nitrocellulose Transfer Membrane (Schleicher & Schuell, Keene, New Hampshire, USA). Following transfer, the membranes were subjected to standard blocking and incubation procedures, and incubated with specific monoclonal and polyclonal antibodies. After washing, membranes were incubated with HRP-labeled secondary antibody, treated with ECL reagent for 1 min, exposed to X-ray film, and developed.

PKC chimeras

PKC chimeras were generated by exchanging the regulatory and catalytic domains of PKC α and PKC δ (Acs *et al.* 1997a), and were kindly supplied by Dr Peter Blumberg (NCI, NIH, Bethesda, MD, USA). PKC α/δ refers to the chimera with the PKC α regulatory domain and the PKC δ catalytic domain, and PKC δ/α refers to the reciprocal chimera. The PKC cDNAs were subcloned into the metallothionein promoter-driven eukaryotic (epsilon MTH) expression vector (MTH) plasmid generating PKC chimeras with a C-terminal ϵ tag. The expression of these chimeras and their activities were described (Blass *et al.* 2002). In this study, we used L8 cells stably overexpressing the PKC $\alpha\delta$ and PKC $\delta\alpha$ chimeras and the control MTH plasmid (Olah *et al.* 1994). The L8 cell line has a low endogenous expression level of PKC δ protein (M Horovitz-Fried & SR Sampson, unpublished observations). Cells were grown in DMEM supplemented with 15% FCS and G418 (Calbiochem, San Diego, CA, USA).

Peptide expression and purification

(His) $_6$ -tagged fusion peptides corresponding to 41 amino acids (amino acids 943–984) of the juxtamembrane region of IR ((His) $_6$ JM) or 86 amino acids (amino acids 1245–1331) of the carboxyl-terminal region of IR ((His) $_6$ CT) were generated in bacteria and purified over ProBond Ni $^{2+}$ beads (Invitrogen) as described by Paz *et al.* 2000. Peptide conjugated beads were stored at 4 °C for the short term until use.

Precipitation of proteins with IR domain peptides

Crude lysate (300 μ g) was incubated with 20 μ l peptide-conjugated beads for 2 h. After washing, sample buffer was added and proteins that had been bound to beads were loaded on an SDS/PAGE gel for further analysis.

Immunoprecipitation

Cell lysates containing equal amounts of protein were incubated with the relevant antibody for 1 h, following which protein A/G agarose beads were added and samples incubated overnight at 4 °C. Precipitates were washed 4 $\times$ with NP-40 buffer and subsequently resuspended in SDS-PAGE sample buffer. Samples were denatured at 90 °C for 4 min and separated by SDS-PAGE.

PKC activity assay

Specific PKC activity was determined in freshly prepared immunoprecipitates (utilizing anti-PKC ϵ

antibodies) from muscle cultures following appropriate treatments, as described (Braiman *et al.* 1999b, Rosenzweig *et al.* 2002). The lysates were prepared in RIPA buffer without NaF. Activity was measured using the SignaTECT Protein Kinase C Assay System (Promega). The kit contains phosphatidylserine and diacylglycerol and utilizes neurogranin as a substrate.

Glucose uptake

Glucose transport was evaluated by measuring 2-deoxy-D-glucose uptake as described (Braiman *et al.* 1999a, 2001a, Rosenzweig *et al.* 2004).

Statistical analysis

In all graphs, values are presented as means $\pm$ S.E.M. Statistical differences between the treatments and controls were evaluated by the Student's *t*-test.

Results

We have shown that activation of PKC δ in skeletal muscle by insulin is associated with a rapid increase in the physical association of PKC δ with IR. This increase in association occurs within 1–5 min in primary cultures of mature rat skeletal myotubes and is associated with regulatory effects on IR internalization and phosphorylation (Braiman *et al.* 2001b). In the current study, we utilized cells from the L6 and L8 rat skeletal muscle cell lines and have initially confirmed this effect in myoblasts (Fig. 1). PKC δ was immunoprecipitated from mature L6 and L8 myotubes following stimulation with insulin for varying times. As shown, PKC δ is constitutively associated with IR in both cell lines under basal conditions, and insulin stimulation causes an increase in association beginning as early as 1 min and reaching a peak at 5 min (Fig. 1A). Similar results were obtained in studies in which IR was immunoprecipitated from lysates of control and insulin-stimulated cells, and immunoblotting was performed with specific anti-PKC antibodies (Fig. 1B). As reported for skeletal muscle in primary culture (Braiman *et al.* 2001b), PKCs α , β II, ϵ , and ζ were not found to associate with IR in L6 or L8 myotubes under either basal or insulin-stimulated conditions (not shown, but see Fig. 1D and (Rosenzweig *et al.* 2002)).

PKC δ preferentially binds to the JM domain of IR

The JM and CT domains of IR have been shown to interact differentially with certain substrates. Thus, Shc was shown to specifically interact with the CT peptide, whereas IRS-1 was found to specifically interact with

Figure 1 Insulin increases association between IR and PKC δ . (A and B) Western blots of insulin-induced co-precipitation of IR with PKC δ in L6 and L8 myotubes (A) and myoblasts (B). Studies were performed on cells transferred to serum-free, low glucose EM, 24 h before experiments were conducted. PKC δ was immunoprecipitated from lysates of untreated cultures (0), or insulin-stimulated cultures treated for the designated times (1, 5 min) with specific anti-PKC δ antibodies. Immunoprecipitates were separated by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with specific anti-IR β antibodies. Upper blot shows co-precipitation of PKC δ with IR. The lower blot shows PKC δ to confirm equal loading of protein. The data presented are representative of four separate experiments. In all figures, CV, cells transfected with control vector only; δ/α , PKC δ regulatory/PKC α catalytic chimera; and PKC α/δ , PKC α regulatory/PKC δ catalytic chimera. (C–E) Insulin-stimulated PKC δ preferentially binds to the JM segment of IR. (C) Extracts were incubated with 10 μ g JM and CT purified peptide for 2 h. Samples were then incubated with Ni $^{++}$ -conjugated beads for 30 min and washed according to the manufacturer's instructions. Precipitates were subjected to SDS/PAGE and immunoblotted with anti-PKC δ antibodies. The lower blots show the relative amounts of JM and CT domains. The data are representative of five separate experiments. (D) Lysates from 6-day-old cultured myotubes treated with insulin for the times indicated were incubated with Ni $^{++}$ -conjugated beads as in C. Precipitates were subjected to SDS/PAGE and immunoblotted with anti-PKC δ (upper blot) and anti-PKC α (middle blot) antibodies. The middle blot shows lack of binding of PKC α under either basal or insulin-stimulated conditions. Lysates immunoblotted with anti His-tag antibodies, showing the relative amounts of JM and CT segments are shown in the lower blot. The data are representative of five separate experiments. (E) Graph of densitometry measurements made in (D). Each bar represents the mean $\pm$ S.E.M. of values obtained in the five experiments (* P < 0.05 versus CV at 0 time; ** P < 0.01 versus corresponding CV and CT).

the JM polypeptide (Paz *et al.* 1996, 1997, 1999). To determine whether PKC δ might also display preferential binding to one or another of the IR domains, we utilized polypeptides of 41 and 86 amino acid lengths, corresponding to the Arg 943-Lys984 (JM) and Leu1245-Asn1331 (CT) domains of the IR respectively. These segments were produced as epitope-tagged fusion peptides containing at their amino terminus a His sequence, fused in-frame to an epitope for the T7Tag antibody (Novagen, Madison, WI, USA).

As shown in Fig. 1C, under basal conditions, PKC δ associates primarily with the JM domain of IR, although some PKC δ also associates with the CT domain.

Insulin induces a selective increase in PKC δ binding to the JM domain of IR

We next sought to determine whether the increase in PKC δ –IR binding in response to insulin stimulation occurs in both domains or is selective for either one or

another. In these studies, mature L6 was stimulated with insulin (100 nM) for 1–5 min, following which PKC δ was immunoprecipitated from whole cell lysates. The stimulated PKC δ was examined for binding to the JM and CT domains. Examples of results of these studies are illustrated in Fig. 1D and E, which shows that PKC δ from insulin-stimulated cells binds selectively to the JM domain. Figure 1D (middle blot) also shows that similar to IR, neither the JM nor CT domain binds detectable amounts of PKC α (or other isoforms – not shown). This confirms for L8 (and L6) myotubes earlier findings obtained in studies on mature skeletal muscle (both rat and mouse) in primary culture that IR selectively binds PKC δ is selective for under both basal and insulin-stimulated conditions (Braiman *et al.* 2001b, Rosenzweig *et al.* 2002).

The relative importance of the JM and CT domains of IR in insulin-induced IR–PKC δ association was further evaluated in 3T3 cells expressing normal hIR or IR in which either the CT (Δ CT) or JM (Δ JM) domains had been deleted (Mosthaf *et al.* 1995). In these studies, we found that in cells in which the CT domain of IR had been deleted, basal binding of PKC δ to IR was actually

higher than in cells expressing normal IR and insulin further increased this association (Fig. 2). In hIR-JM deleted cells, there was a low level of basal association between IR and PKC δ , and this was not increased by insulin (not illustrated).

IR binds both the PKC δ/α and PKC α/δ chimeras

In order to further clarify the relationship between IR and PKC δ , we attempted to determine whether one or the other of the regulatory or catalytic domains of PKC δ preferentially binds to IR. For this purpose, special cell lines of L8 skeletal muscle cells were developed in which PKC α –PKC δ chimeras of the respective regulatory and catalytic domains were stably expressed. The preparation and use of these chimeras in other systems have been reported (Acs *et al.* 1997b, Blass *et al.* 2002). The assembled proteins are composed of either the PKC α regulatory–PKC δ catalytic chimera (PKC α/δ) or the PKC δ regulatory–PKC α catalytic (PKC δ/α) chimera. In addition, we developed cell lines in which reassembled PKC δ/δ and PKC α/α constructs were stably expressed. The PKC chimeras were tagged with a PKC ϵ epitope, which is recognized by anti-PKC ϵ antibody used for western blotting. We have already determined that IR does not bind PKC ϵ or PKC α under either basal or insulin-stimulated conditions in these or other skeletal muscle cells (rat and mouse skeletal muscle in primary culture; mouse skeletal muscle *in vivo*). Western blotting in cells expressing reconstructed PKCs α/α and δ/δ was done with anti-PKC δ antibodies. Figure 3A shows the levels of over-expressed PKC α/δ and PKC δ/α chimeras and PKC δ (in the α/α and δ/δ cells). The cells express similar high amounts of each of the chimeras. In the current study, IR was immunoprecipitated from lysates of control and insulin-stimulated cells, and following SDS-PAGE, immunoblotted with anti-PKC ϵ antibodies. Interestingly, as seen in Fig. 3B and C, whereas both the PKC δ/α and the PKC α/δ chimeras display detectable levels of binding to immunoprecipitated IR and increased binding following insulin stimulation, the increase in binding of the PKC α/δ chimera in response to insulin was greater than that of the PKC δ/α chimera. The figure also shows that the association of PKC δ with IR under basal conditions in cells expressing the re-assembled PKC δ/δ was greater than that in either control cells or in cells expressing the reconstructed PKC α/α . This is in agreement with our earlier studies on skeletal muscle cells in primary culture (Braiman *et al.* 2001b). We next attempted to determine whether the regulatory and catalytic domains might have preferential binding to the JM and CT domains of IR. In these studies, L8 myotubes expressing the PKC δ/α or PKC α/δ chimeras were stimulated with insulin (100 nM) for 1–5 min,

Figure 2 Insulin increases IR–PKC δ association in 3T3 cells expressing human insulin receptor (hIR) and in cells expressing CT domain-deleted hIR (Δ CT). 3T3 fibroblasts expressing hIR and CT-deleted hIR were treated or not with insulin for the designated times. Following treatment, PKC δ (A) or IR (B) was immunoprecipitated with specific anti-PKC δ or anti-IR antibodies. Immunoprecipitates were subjected to SDS/PAGE, transferred to nitrocellulose and immunoblotted with anti-IR (A) or anti-PKC δ (B) antibodies (upper blot), and anti-PKC δ or anti-IR antibodies (lower blots). (C) Densitometry measurements made on western blots in experiments on immunoprecipitated IR (see B). Values were normalized to levels of IR. Each bar represents the mean $\pm$ S.E.M. of values obtained from three separate experiments (* P < 0.05, Δ CT versus hIR).

Figure 3 Insulin increases the binding of IR to both δ/α and α/δ PKC chimeras. L8 cells stably expressing the designated chimeras were treated as in Fig. 1 (CV, cells transfected with control vector only; δ/α , PKC δ regulatory/PKC α catalytic chimera; α/δ , PKC α regulatory/PKC δ catalytic chimera). (A) Western blot of expression of PKC δ/α and α/δ chimeras tagged with PKC ϵ , and of PKC δ in reconstituted PKC α/α and δ/δ cells (see Materials and methods). (B) Western blot showing IR–PKC δ binding in control and insulin-stimulated chimeras and cells expressing reconstructed PKC α/α or PKC δ/δ cells. IR was immunoprecipitated from cell lysates the various cells with specific anti-IR β antibodies. Immunoprecipitates were subjected to SDS/PAGE, transferred to nitrocellulose, and immunoblotted with anti-PKC δ antibodies (upper blot) or anti-IR antibodies (lower blot). (C) Densitometry measurements made on western blots obtained in experiments in C. Values were normalized to levels of IR. Each bar represents the mean $\pm$ S.E.M. of values obtained from three separate experiments. (D) Insulin-induced binding of PKC δ to the JM domain of IR is greater in cells expressing the PKC α/δ chimera than in cells expressing the PKC δ/α chimera. Extracts from cells stimulated or not with insulin were incubated with 10 μ g JM purified peptide for 2 h as in Fig. 1. Precipitates were subjected to SDS/PAGE and immunoblotted with anti-PKC ϵ antibodies. The lower blots show the relative amounts of His-tagged JM domain. The data are representative of five separate experiments. (E) Densitometry measurements made on western blots in D. Values were normalized to levels of JM. Each bar represents the mean $\pm$ S.E.M. of values obtained from three separate experiments. (* $P < 0.05$ versus CV; ** $P < 0.01$ versus CV and δ/α chimera).

following which PKC ϵ was immunoprecipitated from whole cell lysates. These stimulated PKC chimeras were examined for binding to the JM domain. Examples of results of these studies are illustrated in Fig. 3D and E. Both the PKC α/δ and the PKC δ/α chimeras bind to the JM as detected by immunoblotting with the anti-PKC ϵ antibody. However, the binding of the PKC α/δ chimera to JM was significantly greater than that of the PKC δ/α chimera. Moreover, insulin increased the binding of each to the JM but not to the CT domain, as also was shown in Fig. 1 with regard to PKC δ .

The different responses of the two chimeras with regard to the binding to both IR and to its JM domain suggest that IR signaling in the two types of cells may show fundamental changes. Accordingly, we examined different aspects of IR signaling in the various chimeras.

Tyrosine phosphorylation of IR and IRS-1 by insulin is differentially affected in PKC δ/α and PKC α/δ chimeras

We earlier showed that overexpression of PKC δ increased basal IR tyrosine phosphorylation (Braiman *et al.* 2001b). We first examined tyrosine phosphorylation of IR and IRS-1 in response to insulin in the cells expressing the different chimeras. The results of the studies on tyrosine phosphorylation of IR in response to insulin are summarized in Fig. 4A and B. IR was immunoprecipitated from control and insulin-stimulated cells and immunoblotted with anti-phosphotyrosine antibodies. As can be seen, IR phosphorylation in the PKC δ/α chimera was increased compared to control cells. In contrast, whereas basal IR phosphorylation was essentially unchanged in the PKC α/δ chimera compared to control, insulin-induced tyrosine phosphorylation was nearly completely abrogated. The pattern of IR tyrosine phosphorylation in

Figure 4 Insulin-induced tyrosine phosphorylation of IR and IRS-1 is differentially affected in cells expressing the PKC δ/α and α/δ chimera. L8 cells stably expressing the appropriate chimera were treated as in Fig. 1. In all figures, CV, cells transfected with control vector only; δ/α , PKC δ regulatory/PKC α catalytic chimera; and PKC α/δ , PKC α regulatory/PKC δ catalytic chimera. (A) IR was immunoprecipitated from lysates with specific anti-IR antibodies. Immunoprecipitates were subjected to SDS/PAGE and immunoblotted with anti-phosphotyrosine antibodies (upper blot) or anti-IR antibodies (lower blot). (B) Graph showing results of densitometry measurements made on western blots. Values were normalized to levels of IR. Each bar represents the mean $\pm$ S.E.M. of values obtained from three separate experiments (*significantly different from α/δ chimera). (C) IRS-1 was immunoprecipitated from lysates with specific anti-IRS-1 antibodies. Immunoprecipitates were subjected to SDS/PAGE and immunoblotted with anti-phosphotyrosine antibodies (upper blot) or anti-IRS-1 antibodies (lower blot). (D) Densitometry measurements made on western blots. Values were normalized to levels of IRS-1. Each bar represents the mean $\pm$ S.E.M. of values obtained from three separate experiments (* $P < 0.01$ versus α/δ chimera).

response to insulin in PKC δ/δ and PKC α/α cells did not appear to differ from that in control and PKC α/δ cells (not shown).

The different effects of insulin on IR tyrosine phosphorylation in cells expressing the different chimeras suggest that downstream insulin signaling might also differ in these cells compared to control. In order to investigate this possibility, we examined possible differences in responses of downstream proteins to insulin among the chimeras. In one series of experiments, we analyzed the effects of insulin on IRS-1. We immunoprecipitated IRS-1 from control and insulin-stimulated cells expressing the different vectors, and immunoblotting was performed with anti-phosphotyrosine antibodies. These results are summarized in Fig. 4C and D. IRS-1 tyrosine phosphorylation in response to insulin in the PKC δ/α chimera was increased over that in control cells, whereas that in cells expressing the PKC α/δ chimera was slightly reduced compared to control. Tyrosine phosphorylation of IRS-1 induced by insulin in the chimeras expressing PKC α/α or PKC δ/δ was not detectably different from that in cells transfected with control vector (CV; not shown).

Serine phosphorylation of IRS-1 in response to insulin is differentially affected in PKC δ/α and PKC α/δ chimeras

The results showing a decrease in insulin signaling to IRS-1 in the PKC α/δ chimera compared to the control or PKC δ/α -expressing cells suggest that an inhibitory effect on IR signaling might be dominant in the PKC α/δ cells and not the others. This effect could also result in part from differences in serine phosphorylation of IRS-1 in response to insulin stimulation. To investigate this possibility, we examined the serine phosphorylation state of IRS-1 in the different cells before and following insulin stimulation. We prepared whole cell lysates of control and insulin-stimulated cultures of each of the clones, as in the foregoing experiments. In order to determine that IRS-1 is indeed phosphorylated, we performed a series of experiments in which immunoblotting with antibodies directed against IRS-1 Ser307 of control and insulin-stimulated chimeras was performed. This serine residue has been suggested to be a specific inhibitory target for PKC isoforms, possibly in response to insulin (Werner *et al.* 2004, Mussig *et al.* 2005). Figure 5A and B show that in CV cells, IRS-1 was constitutively phosphorylated on

Figure 5 Phosphorylation of IRS-1 Ser307 and PKC activity are differentially affected in PKC δ/α and PKC α/δ chimeras. L8 cells stably expressing the PKC δ/α or the PKC α/δ chimera were treated as in Fig. 1. In all figures, CV, cells transfected with control vector only; δ/α , PKC δ regulatory/PKC α catalytic chimera; and PKC α/δ , PKC α regulatory/PKC δ catalytic chimera. (A) Following insulin treatment and SDS/PAGE, immunoblotting was performed with either anti-phospho-IRS-1 (Ser 307) antibodies (upper blot) or anti-actin antibodies (lower blot). (B) Densitometry measurements made on western blots. Values were normalized to levels of actin. Each bar represents the mean $\pm$ S.E.M. of values obtained from three separate experiments (*significantly different from control CV; ** $P < 0.01$ versus corresponding CV). (C) Fresh lysates from control and insulin-stimulated cells stably expressing the appropriate chimera were treated as in Fig. 1. Seven hundred and fifty micrograms protein was submitted to IP using anti-PKC ϵ antibodies. Immunoprecipitates were then used in an activity assay, following the manufacturer's instructions as described in Materials and methods. (* $P < 0.05$ versus control CV; ** $P < 0.05$ versus δ/α chimera).

Ser307, and this phosphorylation was increased by insulin. Interestingly, in cells expressing the PKC δ/α chimera, basal phosphorylation on Ser307 appeared to be reduced, and insulin failed to increase the level of Ser307 phosphorylation. In contrast, Ser307 phosphorylation in PKC α/δ -expressing cells was significantly increased compared to that in cells expressing either the PKC δ/α chimera or the empty vector. The results obtained in PKC δ/δ cells were qualitatively similar to those in the PKC δ/α cells, while those in PKC α/α were similar to those in controls (not shown).

PKC activity is increased in the PKC chimeras

The different levels of IRS-1 serine phosphorylation in the chimeras compared to control cells suggest that PKC activities might also differ. Therefore, we measured PKC activity under basal and insulin-stimulated conditions for each of the chimeras. Fresh lysates from insulin-stimulated cells were prepared and submitted to immunoprecipitation with PKC ϵ antibody to specifically bring down the ϵ -tagged chimeras. The immunoprecipitation complex was then submitted to an activity assay (as described in Materials and methods). Results are summarized in Fig. 5C. In CV

cells, insulin caused a fourfold increase in insulin-stimulated PKC ϵ by 5 min. This agrees with unpublished data in our laboratory, and is consistent with the increase obtained in insulin-stimulated PKC δ activation. PKC activity in cells expressing either the PKC δ/α or the PKC α/δ chimeras was increased by insulin. However, both basal and insulin-stimulated activities of the cells expressing the PKC α/δ chimera were greater than that of the cells expressing the PKC δ/α chimera. Activity in PKC α/α did not differ from that in CV cells, whereas that in PKC δ/δ cells was similar to that in PKC δ/α chimera-expressing cells (not shown).

Activation of downstream proteins by insulin is different in PKC δ/α and PKC α/δ chimeras

The different patterns of insulin-induced tyrosine and serine phosphorylations of IRS-1 in the cells expressing the different chimeras suggest that responses of other downstream proteins to insulin might also be altered. An immediate downstream target of PI3K is PKB. One indication of PKB activation is phosphorylation on threonine residues. Therefore, we compared threonine phosphorylation of PKB induced by insulin in cells expressing the PKC δ/α with that in cells expressing the PKC α/δ chimeras. Whole cell lysates were prepared

Figure 6 Insulin-induced threonine phosphorylation of PKB, serine phosphorylation of GSK3, and glucose uptake are differentially affected in PKC δ/α and PKC α/δ chimeras. In all figures, CV, cells transfected with control vector only; δ/α , PKC δ regulatory/PKC α catalytic chimera; and PKC α/δ , PKC α regulatory/PKC δ catalytic chimera. (A) Western blots showing equal expression levels of PKB (upper blot) and GSK (lower blot) in cells expressing the different chimeras. (B and C) L8 cells stably expressing the appropriate chimera (B, reconstructed α/α and δ/δ ; C, PKC δ/α , PKC α/δ) were treated as in Fig. 1. Cell lysates from control and insulin-stimulated cells were subjected to SDS/PAGE and immunoblotted with anti-phosphoPKB threonine antibodies (upper blot) or anti-phosphoGSK antibodies (middle blot). The lower blot in each figure shows actin levels verifying equal loading of protein. (D) Densitometry measurements made on western blots of p-PKB and p-GSK in experiments as in C. Values were normalized to levels of actin. Each bar represents the mean $\pm$ S.E.M. of values obtained from three separate experiments. (** $P < 0.05$ versus CV or δ/α chimera; * $P < 0.05$ versus δ/α chimera). (E) Insulin stimulated glucose uptake in cells expressing the PKC δ/α chimera is reduced compared to control and PKC δ/α -expressing cells. Glucose uptake was measured, as described in Materials and methods, in untreated (gray bars) and insulin-stimulated (black bars) control (CV) cells and in cells expressing the PKC δ/α and PKC α/δ or reconstructed PKC α/α and PKC δ/δ chimeras (insulin treatment 30 min). Data represent the mean $\pm$ S.E.M. of triplicate measurements obtained in four different experiments ($n = 12$). The data are presented as the fold increase above basal level of glucose uptake in untreated cells. Basal and insulin-stimulated glucose uptake in the PKC δ/δ and PKC δ/α chimeras, while not different from each other, were greater than control CV ($P < 0.05$), and in the PKC α/δ chimera not significantly different from those in CV control.

from control and insulin-stimulated cells expressing the different chimeras. The lysates were then subjected to SDS-PAGE and immunoblotting with specific anti-phosphoPKB Thr308 antibodies. As shown in Fig. 6A, protein levels of PKB and GSK3 were similar in each of the PKC chimeras. Figure 6B shows that insulin-induced phosphorylation of PKB and GSK in cells expressing re-assembled PKC δ/δ was slightly

higher, and that in cells expressing PKC α/α was similar to that in control cells. Figure 6C and D show that insulin-induced phosphorylation of PKBThr308 and GSK-3Ser9 was slightly higher in cells expressing the PKC δ/α chimera compared to control. On the other hand, phosphorylation of PKBThr308 and GSK-3Ser9 was strongly and significantly reduced in cells expressing the PKC α/δ chimera.

Insulin-induced 2-DOG uptake is different in PKC δ/α and PKC α/δ chimeras

Our results so far demonstrate that insulin signaling to PKB and GSK3 is increased in PKC δ/α -expressing cells and decreased in PKC α/δ -expressing cells. As PKB is also considered an important component in signaling to glucose uptake, we next measured the uptake of 2-DOG in response to insulin stimulation of L8 myoblasts expressing the different PKC chimeras. Studies were performed on undifferentiated L8 myoblasts because cells expressing the different

chimeras displayed varying rates of differentiation. This prevented us from performing glucose uptake studies on the same day for each type. Myoblasts have been used as a model for glucose uptake studies (Randhawa *et al.* 2000, Sweeney *et al.* 2004, Aga-Mizrachi *et al.* 2008). The results are summarized in Fig. 6E. For comparison, glucose uptake in non-transfected L8 myoblasts is included. Basal and insulin-stimulated glucose uptake in CV-expressing cells was slightly but significantly lower than that in L8 myoblasts. In myoblasts expressing the CV, insulin induced a slight (20–40%) but significant increase in glucose

Figure 7 Insulin-induced interactions between PKC δ and Src, between IR and Src, and Src activity are differentially affected in PKC δ/α and PKC α/δ chimeras. L8 cells stably expressing the PKC δ/α or the PKC α/δ chimera were treated as in Fig. 1. In all figures, CV, cells transfected with control vector only; δ/α , PKC δ regulatory/PKC α catalytic chimera; and PKC α/δ , PKC α regulatory/PKC δ catalytic chimera. (A and B) PKC–Src binding. (A) Following insulin treatment and SDS/PAGE, cells were immunoprecipitated with anti-PKC ϵ antibodies, and immunoblotting was performed with either anti-Src (upper blot) or anti-actin antibodies (lower blot). (C and D) IR–Src binding. Following insulin treatment and SDS/PAGE, cells were immunoprecipitated with anti-IR antibodies, and immunoblotting was performed with either anti-Src (upper blot) or anti-IR (lower blot) antibodies. (B) and (D) are graphs of densitometry measurements made on western blots. Values were normalized to levels of the appropriate protein. Each bar represents the mean $\pm$ S.E.M. of values obtained from three separate experiments (*significantly different from corresponding CV). (E and F) Insulin-induced Src^{Y529} dephosphorylation. (E) is a representative western blot of insulin-induced dephosphorylation of Src^{Y529} in L8 cells expressing the different chimeras. (F) is a plot of densitometry measurements made on western blots. Values were normalized to levels of the appropriate protein. Each bar represents the mean $\pm$ S.E.M. of values obtained from three separate experiments. Src^{Y529} phosphorylation was decreased (increased Src activity) by insulin in cells expressing CV and the δ/α and δ/δ chimeras and was increased (decreased Src activity) in cells expressing the α/δ and α/α chimeras. (* $P < 0.05$, ** $P < 0.01$, versus time 0). Clear bars, time 0; gray bars, insulin 1 min; black bars, insulin 5 min.

uptake. In cells expressing either the PKC δ/δ or the PKC δ/α chimera, basal glucose uptake was significantly higher than that in control, and insulin induced a slight but significant increase in glucose uptake compared to control. In contrast, whereas basal glucose uptake in PKC α/δ -expressing cells was only slightly lower than that in controls, insulin-induced glucose uptake was almost totally abrogated in these cells.

Interaction of Src with PKC δ and IR is different in PKC δ/α and PKC α/δ chimeras

The data shown so far demonstrate that insulin signaling from IR tyrosine phosphorylation to glucose uptake is up-regulated in cells expressing the PKC δ/α chimera, whereas it is strongly downregulated in cells expressing the PKC α/δ chimera. The increased IR tyrosine phosphorylation in the δ/α cells is unlikely to be a direct effect of the PKC δ regulatory domain and probably results from involvement of a tyrosine kinase. It was reported that Src tyrosine kinase upregulates IR tyrosine phosphorylation, and we have shown that insulin activates Src and also induces Src–PKC δ association. Moreover, Src is involved in insulin-induced tyrosine phosphorylation and activation of both PKC α and PKC δ (Rosenzweig *et al.* 2004, Cipok *et al.* 2006). We, therefore, examined the possibility that PKC δ –Src and IR–Src interactions may be different in the two chimeras. To this end, we immunoprecipitated PKC ϵ or IR from the different chimeras before and after treatment with insulin. Then, following SDS-PAGE and transfer, immunoblotting was performed with anti-Src antibodies. As shown in Fig. 7A and B, in control (CV transfected) cells, insulin induced an increase in PKC δ –Src association within 1 min, and this increased further by 5 min, similar to our earlier findings (Rosenzweig *et al.* 2004). In cells expressing the PKC δ/α chimera, basal PKC δ –Src association was increased over that in CV cells, and insulin increased this association even further at 1 and 5 min. Cells expressing the PKC α/δ chimera also displayed higher basal PKC δ –Src association. This association was also increased after 1 min by insulin but did not increase with time. Insulin-induced PKC δ –Src association after 5 min was higher in the δ/α than in the α/δ chimera. These data suggest that the PKC δ regulatory domain may be important in regulation of PKC δ –Src interactions.

We next examined the effects of insulin on IR–Src association in cells expressing the different chimeras. As shown in Fig. 7C and D, insulin increased IR–Src association in control cells, as we have earlier reported (Rosenzweig *et al.* 2004). Constitutive IR–Src association in both PKC δ/α - and PKC α/δ -expressing cells was higher than that in control cells; however, effects of

insulin on these associations were strikingly different. Thus, elevated IR–Src association in the cells expressing the PKC δ/α chimera remained relatively constant with insulin stimulation, whereas IR–Src association in the cells expressing the PKC α/δ chimeras was decreased. Finally, we examined the effects of insulin on phosphorylation of Src tyrosine 529 (Src^{Y529}). A decrease in phosphorylation is an indication of Src activation (Harder *et al.* 1998, Bjorge *et al.* 2000). The results are summarized in Fig. 7E and F. In both CV and PKC δ/α -expressing cells, insulin induced a decrease in Src^{Y529} phosphorylation. In contrast, in cells expressing the PKC α/δ chimera, Src^{Y529} phosphorylation was increased, indicating that activity of Src tyrosine kinase actually decreased. Src phosphorylation in PKC α/α -expressing cells was also decreased, although with a different time course, whereas that in PKC δ/δ -expressing cells did not appear to be significantly changed. These findings indicate that the PKC δ and PKC α regulatory domains have different roles in regulation of Src and its effects on IR signaling.

Discussion

In an earlier series of studies, we showed that PKC δ is activated by insulin upstream of PI3K (Braiman *et al.* 1999b) and is tyrosine phosphorylated and activated via a Src tyrosine kinase-dependent mechanism (Rosenzweig *et al.* 2004). In addition, PKC δ was found to be a positive regulator of insulin signaling and uniquely regulated by insulin. Thus, overexpression of WTPKC δ in primary skeletal muscle cells increased GLUT4 translocation and glucose uptake in the absence of insulin stimulation, and inhibition of PKC δ by pharmacological means or by expression of a kinase-dead dominant-negative construct abrogated insulin-induced GLUT4 translocation and glucose uptake (Braiman *et al.* 1999a). In addition, PKC δ was induced by insulin to directly associate with IR and regulate insulin-induced tyrosine phosphorylation and internalization of IR (Braiman *et al.* 2001b); overexpression of PKC δ increased IR tyrosine phosphorylation and induced internalization, whereas PKC δ blockade prevented IR tyrosine phosphorylation and internalization. The binding of PKC δ to IR is one of the earliest effects of insulin stimulation of skeletal muscle and is unique to PKC δ . Insulin not only stimulates PKC δ but also regulates its expression and protein levels (Horovitz-Fried *et al.* 2006a,b, 2008, Horovitz-Fried & Sampson 2007). In addition, studies in intact animals have shown that elevated PKC δ activity is involved in the effect of exercise to prevent the development of diabetes in diabetes-prone animals (Heled *et al.* 2002, 2003). These findings present an apparent paradox. On the one hand, PKC δ is a serine–threonine kinase, which

should act to downregulate IR signaling; on the other hand, PKC δ acts to upregulate IR signaling.

The results of the current study offer a reasonable explanation to resolve this paradox, namely that the regulatory and catalytic domains of PKC δ serve independent functions related to the physical binding between IR and PKC δ and to the effects of PKC δ on IR signaling. Thus, insulin-induced binding of PKC δ to IR was higher in cells expressing the PKC α /PKC δ chimera than in cells expressing any of the other chimeras. In contrast, cells expressing the PKC δ / α chimera displayed higher levels of IR and IRS-1 tyrosine phosphorylation and increased signaling to PKB/Akt, GSK-3, and glucose uptake. PKC α does not bind to IR, either under basal or insulin-stimulated conditions (Fig. 1D and Braiman *et al.* 2001b, Rosenzweig *et al.* 2002, Cipok *et al.* 2006), and this difference could be due to some change imparted to the PKC δ catalytic domain to increase its affinity for IR when activated. Alternatively, this could involve an effect secondary to the lack of the PKC regulatory domain, which might interact with an additional protein such as Src, which binds to both IR and PKC δ (Rosenzweig *et al.* 2004).

The differential effects of the two chimeras on the insulin signaling cascade lend further support to the proposal that the regulatory domain of PKC δ is responsible for the upregulation of insulin signaling by this isoform. However, as PKC δ is a serine–threonine kinase, it itself cannot increase IR and IRS-1 tyrosine phosphorylation, suggesting that a tyrosine kinase must be involved; one such possibility is Src tyrosine kinase. In accordance with this, we reported that insulin-activated PKC δ associates with Src tyrosine kinase and increases its activity (Rosenzweig *et al.* 2004). Furthermore, inhibition of Src reduces insulin-stimulated Src–PKC δ association, PKC δ tyrosine phosphorylation and PKC δ activation. Inhibition of Src also decreases insulin-induced IR tyrosine phosphorylation, IR–PKC δ association, and association of Src with both PKC δ and IR. Moreover, Src tyrosine kinase was earlier shown to catalyze IR tyrosine phosphorylation (Yu *et al.* 1985). Our current results on interaction of Src tyrosine kinase with PKC δ and IR in the different chimeras support this role for Src, and further provide a plausible explanation for the upregulation of IR signaling in the PKC δ / α -expressing cells compared to the downregulation in the PKC α / δ -expressing cells. Thus, i) PKC δ –Src association was higher in the PKC δ / α -expressing cells than in the PKC α / δ -expressing cells; ii) IR–Src association remained elevated with insulin stimulation in the PKC δ / α -expressing cells, whereas it was decreased by insulin stimulation in the PKC α / δ -expressing cells; and iii) most importantly, insulin decreased phosphorylation of Src^{Y529} (increased activation) in cells expressing the PKC δ / α chimera and increased phosphorylation of Src^{Y529} (decreased

activation) in the cells expressing the PKC α / δ chimera. The increased phosphorylation of Src⁵³⁹ results in inhibition of Src and downregulation of IR signaling, as we have shown here and elsewhere (Rosenzweig *et al.* 2004). We propose, therefore, that differences in PKC δ –Src and IR–Src interactions between the PKC α / δ - and PKC δ / α -expressing cells result in the altered tyrosine phosphorylation and insulin signaling observed in these cells. It was reported that the C2 domain (in the regulatory segment) of PKC δ is a phosphotyrosine-binding domain (Sondermann & Kuriyan 2005); this mechanism for crosstalk between distinct signaling pathways lends strong support to our findings that PKC δ –Src interactions are an integral component of PKC δ regulation of IR signaling. Other studies have shown that the regulatory domain of PKC δ is responsible for the effects of this isoform on regulation of glutamine synthetase expression and cell proliferation by PKC δ in C6 glioma cells and on U87 cell morphology, proliferation, and GFAP expression (Kronfeld *et al.* 2000, Mandil *et al.* 2001, Blass *et al.* 2002).

The increase in insulin-induced phosphorylation of both PKB and GSK in cells expressing the PKC δ / α chimera, as well as the increase in basal glucose uptake, compared to the PKC α / δ chimera, is consistent with the previously shown positive regulatory role for PKC δ in insulin effects. This is in agreement with other studies in our laboratory in which we found that basal glucose uptake was increased significantly in cells overexpressing PKC δ (Braiman *et al.* 1999a) and that PKC δ is involved in insulin-induced regulation of PKB activity via interactions with PDK-1 (phosphoinositide-dependent kinase 1; Brand *et al.* 2006).

We also examined the interaction of PKC δ with specific domains (JM and CT) of IR. Whereas PKC δ did not appear to display any preferential binding to either of these regions under basal non-stimulated conditions, insulin stimulation increased the binding of PKC δ to the JM but not to the CT domain. The JM and CT regions of IR have been shown to be functionally independent entities that contain sufficient structural information to enable independent and direct binding of effector molecules (Paz *et al.* 1996, 1997, 2000). Thus, the JM region was shown to serve as a binding site for IRS-1, as IRS-1 interacts preferentially with the JM peptide and exhibits no significant binding to the CT peptide, similar to our results regarding PKC δ . The preference of binding of PKC δ to the JM domain was supported further by the findings that deletion of the CT domain of IR did not decrease the association between IR and PKC δ , but even increased it under basal as well as in insulin-stimulated conditions. Finally, the binding to the JM region was specific for PKC δ , as binding of PKC α or other isoforms to either the JM or CT domain, either from unstimulated or from

insulin-stimulated cells, could not be detected. These findings are consistent with earlier reports and confirm and extend our earlier results showing that insulin induces a selective interaction between PKC δ and IR.

Our results regarding PKC δ binding to the JM region are compatible with those of Paz *et al.* (1997, 2000), who showed that this region in its non-phosphorylated form is sufficient to promote interactions with downstream effectors such as IRS-1. Phosphorylation of the latter most likely potentiates the interactions, but is not an absolute prerequisite. Other studies indicate that Tyr is poorly phosphorylated *in vivo* (White *et al.* 1988a,b, Tavaré *et al.* 1988, Tornqvist *et al.* 1988), although the JM region is a major site for insulin-stimulated Ser phosphorylation (Feener *et al.* 1993). As we showed, isolated, non-phosphorylated JM regions effectively bind PKC δ in either its inactive or active form.

The results regarding PKC δ -mediated serine phosphorylation of IRS-1 agree with recent studies indicating that this isoform may also be involved in negative regulation of IRS-1, several putative serine sites having been identified (Greene *et al.* 2004, 2006, Waraich *et al.* 2008). Phosphorylation of IRS-1 Ser 307 in the cells expressing the different chimeras appears to support findings in recent studies showing that this serine residue may be a target for insulin-activated PKC isoforms, although in CHO-IR cells Ser24 is also specifically implicated. It was reported that Ser307 was one of 18 sites that could be phosphorylated by PKC δ , and that mutation of serine to alanine on this and two other sites completely abrogated the inhibitory effect of PKC δ on IRS-1 tyrosine phosphorylation *in vitro* (Greene *et al.* 2004, 2006). It should be emphasized that not only was IRS-1 Ser307 phosphorylation increased in PKC α/δ -expressing cells, it was completely abrogated in cells expressing the PKC δ/α chimera. These observations further support the idea that PKC δ upregulates insulin signaling, and that the regulatory domain plays an important role in this process. Nonetheless, the possibility that PKC δ might have a dual effect similar to PKC ζ , see (Liu *et al.* 2001) and also be involved in IRS-1 serine phosphorylation cannot be ruled out.

Our major findings can be summarized as follows. On stimulation by insulin, PKC δ and Src rapidly associate with each other and with the JM domain of IR, coinciding with increased tyrosine phosphorylation of IR, a process in which Src participates. Overexpression of the PKC δ regulatory domain leads to an increase in IR and IRS-1 tyrosine phosphorylation and in IR signaling. In cells in which the PKC δ regulatory domain is downregulated, such as in those expressing the PKC α regulatory/PKC δ catalytic domains, Src interaction with IR is reduced (if not abrogated) even though the PKC α/δ chimera associates with IR, probably via the δ catalytic domain. As a consequence, IR and IRS-1

tyrosine phosphorylations are reduced and serine phosphorylations increased, and IR signaling is downregulated. Thus, our findings strengthen the concept that PKC δ plays an important role in regulation of IR signaling. These effects appear to be dependent on the interactions of the regulatory domain with IR and IRS-1 and involve the participation of additional signaling proteins such as Src tyrosine kinase.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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