# Modulation of human insulin receptor substrate-1 tyrosine phosphorylation by protein kinase C$\delta$

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Non-esterified fatty acid (free fatty acid)-induced activation of the novel PKC (protein kinase C) isoenzymes PKC$\delta$ and PKC$\theta$ correlates with insulin resistance, including decreased insulin-stimulated IRS-1 (insulin receptor substrate-1) tyrosine phosphorylation and phosphoinositide 3-kinase activation, although the mechanism(s) for this resistance is not known. In the present study, we have explored the possibility of a novel PKC, PKC$\delta$, to modulate directly the ability of the insulin receptor kinase to tyrosine-phosphorylate IRS-1. We have found that expression of either constitutively active PKC$\delta$ or wild-type PKC$\delta$ followed by phorbol ester activation both inhibit insulin-stimulated IRS-1 tyrosine phosphorylation *in vivo*. Activated PKC$\delta$ was also found to inhibit the IRS-1 tyrosine phosphorylation *in vitro* by purified insulin receptor using recombinant full-length human IRS-1 and a partial IRS-1–glutathione S-transferase-fusion protein as substrates. This inhibition *in vitro* was not observed with a non-IRS-1 substrate, indicating that it was not the result of a general decrease in the intrinsic kinase activity of the receptor. Consistent with the hypothesis that PKC$\delta$ acts directly on IRS-1, we show that IRS-1 can be phosphorylated by PKC$\delta$ on at least 18 sites. The importance of three of the PKC$\delta$ phosphorylation sites in IRS-1 was shown *in vitro* by a 75–80 % decrease in the incorporation of phosphate into an IRS-1 triple mutant in which Ser-307, Ser-323 and Ser-574 were replaced by Ala. More importantly, the mutation of these three sites completely abrogated the inhibitory effect of PKC$\delta$ on IRS-1 tyrosine phosphorylation *in vitro*. These results indicate that PKC$\delta$ modulates the ability of the insulin receptor to tyrosine-phosphorylate IRS-1 by direct phosphorylation of the IRS-1 molecule.

Key words: insulin resistance, IRS-1 (insulin receptor substrate-1), nPKC (novel protein kinase C), tyrosine phosphorylation.

## INTRODUCTION

Insulin resistance is a major pathophysiological defect leading to obesity and is a good predictor of Type II (insulin-dependent) diabetes mellitus [1]. It has been proposed that a defect in the function of IRS (insulin receptor substrate) proteins plays a key role in the uncoupling of the insulin signal and insulin resistance [2]. IRS proteins link the activation of the insulin receptor at the cell surface to intracellular signalling cascades leading to the biological actions of insulin [3]. The IRS proteins are phosphorylated on tyrosine residues by the activated insulin receptor kinase [3]. Tyrosine-phosphorylated IRS-1 and IRS-2 serve as the major docking proteins for SH2 (Src homology 2) domain-containing proteins [3]. Association of the p85 regulatory subunit of PI3K (phosphoinositide 3-kinase) with tyrosine-phosphorylated IRS-1/IRS-2 results in the membrane localization and activation of the p110 catalytic subunit of PI3K, leading to the generation of PtdIns(3,4,5)$P_3$ and PtdIns(3,4)$P_2$. These lipid products induce many, if not all, of the biological actions of insulin [4] as well as the activation of a number of key signalling kinases, including the Ser/Thr kinase Akt [5].

Some of the same signalling molecules that are involved in the metabolic and mitogenic actions of insulin have also been proposed to play a role both in the feedback inhibition of the insulin signal and in cellular insulin resistance [2,3,6]. The family of PKC (protein kinase C) isoenzymes represents such signalling molecules. On the basis of enzymic properties and relatedness at the amino acid sequence level, the PKC isoenzymes are classified as classical isoenzymes (PKC$\alpha$, PKC$\beta$ and PKC$\gamma$), novel isoenzymes (PKC$\varepsilon$, PKC$\nu$, PKC$\theta$ and PKC$\delta$) and atypical isoenzymes (PKC$\lambda$ and PKC$\zeta$) [7]. Classical PKCs (cPKCs) are activated by phosphatidylserine (PS) and DAG (diacylglycerol) in a $Ca^{2+}$-dependent manner, whereas the novel PKCs (nPKCs) are also activated by PS and DAG, but are insensitive to $Ca^{2+}$ [7]. In contrast, the atypical PKCs (aPKCs) are not activated by PS, DAG and $Ca^{2+}$ [7]. Activation of cPKCs and nPKCs by DAG can be replaced by tumour-promoting phorbol esters such as PMA [7]. A positive regulatory role has been proposed for cPKC, nPKC and aPKC isoenzymes in the insulin-stimulated glucose transport and translocation of GLUT4 to the membrane [8,9]. PKC isoenzymes have also received considerable attention for their negative regulatory role in insulin signalling. Activation of cPKC, nPKC and aPKC isoenzymes inhibits insulin-stimulated signalling in a variety of systems [10,11]. In some systems, activation of PKC isoenzymes leads to enhanced Ser/Thr phosphorylation of the insulin receptor and modulation of insulin receptor internalization [12]. On the other hand, activation of PKC isoenzymes has been shown to enhance IRS-1 Ser/Thr phosphorylation by both direct and indirect mechanisms. For example, the aPKC$\zeta$ isoenzyme has been shown to phosphorylate IRS-1 directly at Ser-318 [13–15], whereas activation of nPKC and cPKC isoenzymes has been shown to lead to MAPK (mitogen-activated protein kinase)-mediated phosphorylation of IRS-1 at Ser-616 and inhibition of insulin-stimulated IRS-1 tyrosine

Abbreviations used: CHO/IR cells, Chinese-hamster ovary cells overexpressing the insulin receptor; DAG, diacylglycerol; DTT, dithiothreitol; GST, glutathione S-transferase; hIR, human insulin receptor; IRS, insulin receptor substrate; MALDI–TOF, matrix-assisted laser-desorption ionization–time-of-flight; MAPK, mitogen-activated protein kinase; NEFA, non-esterified fatty acid (free fatty acid); PH, pleckstrin homology; PI3K, phosphoinositide 3-kinase; PKC, protein kinase C; aPKC, cPKC and nPKC, atypical, classical and novel PKC respectively; PS, phosphatidylserine; PTB domain, phosphotyrosine binding domain; SH2, Src homology 2; wt, wild-type.

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phosphorylation and PI3K activation [16]. (Unless otherwise specified, the sequence numbers shown correspond to the human sequence of IRS-1.)

Recent attention has focused on the role of nPKCs in NEFA (non-esterified fatty acid or free fatty acid)-induced insulin resistance [17–19]. Lipid infusion in rats and humans results in the impairment of insulin-stimulated glucose disposal in muscles, which is associated with a decreased level of insulin-stimulated IRS-1 tyrosine phosphorylation and associated PI3K activity [17,20–22]. Concomitant with the lipid-induced insulin resistance in muscles is the activation of PKC$\theta$ and PKC$\delta$ in rats and humans respectively. Lipid infusion also blocks the insulin-mediated suppression of endogenous glucose production in the liver, with concomitant activation of PKC$\delta$ [23]. Thus PKC$\delta$ appears to be one of the major PKC isoenzymes activated by lipid infusion. The activation of PKC$\delta$ correlates with insulin resistance and decreased level of insulin-stimulated IRS-1 tyrosine phosphorylation and associated PI3K activity, yet the mechanism of action is unknown. PKC$\delta$ may promote enhanced Ser/Thr phosphorylation of IRS-1, which is a negative regulator of insulin signalling. It is possible that this occurs by an indirect mechanism as suggested previously [22]. Alternatively, PKC$\delta$ may directly phosphorylate IRS-1 and inhibit IRS-1 function. To test this hypothesis, we have investigated the inhibition of insulin-stimulated IRS-1 tyrosine phosphorylation by PKC$\delta$ in cell-culture models and PKC$\delta$-mediated phosphorylation *in vitro*.

## EXPERIMENTAL

### Materials

Polyclonal antibodies to IRS-1 and PI3K (p85) and monoclonal 4G10 (P-Tyr) were obtained from Upstate Biotechnology (Lake Placid, NY, U.S.A.). Polyclonal anti-pTyr-941 and anti-pTyr-896 IRS-1 antibodies were obtained from Biosource (Camarillo, CA, U.S.A.), polyclonal anti-GST (glutathione S-transferase) and anti-PKC$\delta$ (rat) antibodies from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.) and monoclonal anti-PY20 (P-Tyr) from Oncogene Research Products (Cambridge, MA, U.S.A.). Cloning enzymes and competent DH5$\alpha$ and BL21DE cells were obtained from Invitrogen (Carlsbad, CA, U.S.A.), plasmid purification kits from Qiagen (Valenca, CA, U.S.A.) and turbo Pfu and Quik Change XL mutagenesis kit from Stratagene (La Jolla, CA, U.S.A.). $Zn^{2+}$-affinity resin (Talon) was obtained from BD Biosciences (Clontech, Palo Alto, CA, U.S.A.) and enhanced chemiluminescence detection reagents from Pierce (Rockford, IL, U.S.A.). Tris/glycine gels, NuPAGE gels and electrophoresis reagents were obtained from Novex/Invitrogen (Carlsbad, CA, U.S.A.) and recombinant PKC$\delta$ and PKC$\theta$ enzymes from Upstate Biotechnology (Lake Placid, NY, U.S.A.). Goat anti-mouse and anti-rabbit peroxidase-conjugated antibodies, monoclonal anti-FLAG, anti-FLAG agarose and other chemicals were obtained from Sigma (St. Louis, MO, U.S.A.).

### Plasmid construction, mutagenesis and recombinant protein production

To make the GST–IRS-1-fusion protein, PCR was used to subclone human IRS-1 cDNA (amino acid residues 288–678) into pGEX 4T-3 in-frame to generate pGEX GST–hIRS-$1^{288-678}$ (where hIR stands for human insulin receptor). GST–hIRS-1 and full-length IRS-1 serine residues in the fusion protein were mutated to alanine residues using the Quik Change and Quik Change XL mutagenesis kits respectively according to the manufacturer's instructions. The plasmids and mutant sites were verified by restriction digestion and sequencing. BL21 DE3 cells transformed with pGEX GST-hIRS-$1^{288-678}$ or pGEX GST-PH-PTB rIRS-$1^{1-309}$ [where PH and PTB stand for pleckstrin homology and phosphotyrosine binding (domains) respectively] [24] were grown to $A_{600}$ 0.8–1.2 at 37 °C in the presence of antibiotic before induction with 0.1 mM isopropyl $\beta$-D-thiogalactoside, followed by shaking at 220 rev./min at room temperature (23 °C) for 3 h. Cultures were centrifuged at 7700 ***g*** for 10 min at 4 °C. The bacterial pellet was stored at − 80 °C before the addition of B-Per Reagent (Pierce) plus 1× protease inhibitors (Calbiochem, San Diego, CA, U.S.A.). After gentle agitation for 10 min, the cells were sonicated 3–6 times on ice for 30–45 s. Cellular debris was removed by centrifugation at 27 000 ***g*** for 15 min at 4 °C. The supernatant was incubated with washed glutathione–agarose at room temperature for 30 min, followed by washing (three times with PBS + protease inhibitors) and elution with 10 mM glutathione in 50 mM Tris (pH 8.0). Fractions containing the GST-fusion protein were pooled and dialysed in PBS buffer containing 2 mM DTT (dithiothreitol) and 0.05 % Tween 20 using Slide-A-Lyzer cassettes (Pierce). BL21-SI cells transformed with pET28 His-PKC$\delta$ [25] were grown, induced, collected and stored as described above. The bacterial pellet was resuspended in 50 mM $NaH_2PO_4$ (pH 8.0), 300 mM NaCl and 10 mM imidazole plus 10 $\mu$g/ml leupeptin and aprotinin. After the addition of 0.75 mg/ml lysozyme, the cells were incubated on ice for 10 min and then sonicated eight times on ice for 30 s. Cellular debris was removed by centrifugation at 12 000 ***g*** for 25 min at 4 °C. The supernatant was applied twice to a $Zn^{2+}$-affinity column (Clontech), followed by washing with 50 mM $NaH_2PO_4$ (pH 8.0), 300 mM NaCl and 20 mM imidazole and elution with 50 mM $NaH_2PO_4$ (pH 8.0), 300 mM NaCl and 150 mM imidazole. The amount of total protein in each fraction was determined using the Bradford assay (Pierce) with BSA as the standard. An equal volume of glycerol was added to each fraction before storage at − 80 °C.

### *In vitro* kinase assays

FLAG–IRS-1 (750 ng) or GST–hIRS-$1^{288-678}$ (1 $\mu$g) and PKC$\delta$ proteins were incubated in 30 $\mu$l of reaction mixture containing 20 mM Mops (pH 7.2), 10 mM $\beta$-glycerophosphate, 1 mM DTT, 0.1 mg/ml PS and 30 nM PMA, and reactions were started by the addition of 0.1 mM ATP/15 mM $MgCl_2$ containing [$\gamma$-$^{32}$P]ATP (specific activity, 3000 c.p.m./pmol). The reaction was terminated after 30 min by the addition of 10 $\mu$l of 4 × SDS sample buffer containing 200 mM DTT, and the sample was heated at 100 °C for 5 min. Control experiments demonstrated that GST is not a substrate for PKC$\delta$.

Alternatively, recombinant FLAG–IRS-1 and GST–hIRS-$1^{288-678}$ proteins were phosphorylated by PKC$\delta$ in the absence of [$\gamma$-$^{32}$P]ATP. The reaction was terminated after 45 min by the addition of ice-cold 15 mM EDTA and 200 mM NaCl, followed by immunoprecipitation for 2 h at 4 °C using anti-FLAG agarose and glutathione–agarose respectively in 50 mM Tris (pH 7.4), 200 mM NaCl and 10 mM EDTA. Bound recombinant proteins were washed twice with 50 mM Tris (pH 7.4) and 200 mM NaCl, once with 50 mM Tris (pH 7.4) and 500 mM NaCl, once with 50 mM Tris (pH 7.4) and 150 mM NaCl and once with 5 mM Tris (pH 7.4) and 75 mM NaCl. The washed agarose-bound IRS-1 proteins were incubated with 30 $\mu$l of activated insulin receptors for 30 min at 30 °C. The reaction was stopped by the addition of 10 $\mu$l of 4 × SDS sample buffer containing 200 mM DTT. Insulin receptors were activated at 4 °C for 1 h by incubation of 70 ng of solubilized hIR-HEK-293 cell membranes, prepared as described previously [26], 20 mM Tris

(pH 7.6), 10 mM $MgCl_2$, 100 μM ATP, 1 mM DTT, 25 μg/ml BSA and 100 nM insulin. Control experiments demonstrated that tyrosine phosphorylation by solubilized hIR-HEK-293 cell membranes was linear with respect to enzyme and substrate.

Insulin receptor kinase activity was determined using the exogenous substrate poly(Glu/Tyr) (4:1; 1 mg/ml) and [$\gamma$-$^{32}$P]ATP (specific activity, 1800 c.p.m./pmol) as described previously [26].

### Phosphorylation site analysis

Recombinant IRS-1 (10 μg) and PKCδ proteins were incubated in 100 μl of reaction mixture containing 20 mM Mops (pH 7.2), 10 mM β-glycerophosphate, 1 mM DTT, 1 mg/ml PS and 0.2 mM PMA, and reactions were started by the addition of 0.10 mM ATP/15 mM $MgCl_2$ containing [$\gamma$-$^{32}$P]ATP (9000 c.p.m./pmol). The reaction was terminated after 30–45 min by the addition of 10 μl of 4 × LDS sample buffer (Invitrogen) containing 40 mM DTT, and the sample was heated at 100 °C for 1 min. After cooling, 4-vinylpyridine was added to a concentration of 0.5 % (v/v), and the samples were placed on a shaking platform for 30 min at room temperature to alkylate cysteine residues. Each reaction was subjected to electrophoresis on a 4–12 % SDS/acrylamide gel and then analysed by autoradiography. The phosphorylated band corresponding to $^{32}$P-labelled IRS-1 was excised from the gel and cut into small pieces. The gel pieces were washed sequentially, for 15 min/wash, on a vibrating platform with 1 ml of water, followed by 1 ml of water/acetonitrile (1:1, v/v), 0.1 M ammonium bicarbonate, 0.2 M ammonium bicarbonate/acetonitrile (1:1, v/v) and finally 100 % (v/v) acetonitrile. The gel pieces were dried in a rotary evaporator and incubated in 0.2 ml of 50 mM ammonium bicarbonate/0.05 % (w/v) Zwittergent 3–16 containing 2 μg of trypsin or endoproteinase Lys-C. After 16 h, the supernatant was removed and the gel pieces were washed for 10 min in 0.2 ml of 50 mM ammonium bicarbonate/0.05 % (w/v) Zwittergent 3–16/0.1 % (v/v) trifluoroacetic acid. The combined supernatants, which contained > 90 % of the $^{32}$P radioactivity, were chromatographed on a Vydac $C_{18}$ column as described in the legend to Figure 3.

$^{32}$P-labelled peptides were analysed at the MRC Protein Phosphorylation Unit at the University of Dundee by MALDI–TOF (matrix-assisted laser-desorption ionization–time-of-flight)-MS on a PerSeptive Biosystems Elite STR mass spectrometer (Framingham, MA, U.S.A.), using α-cyanocinnamic acid as the matrix. Spectra were obtained in both linear and reflector modes. Peptide masses obtained from MALDI–TOF mass spectra in the reflector mode are given as monoisotopic masses, whereas those obtained in the linear mode are given in average mass units. Mass values were used to search against the predicted masses of predicted human IRS-1 tryptic peptides, and candidate peptides were identified. The site of phosphorylation in each HPLC-purified phosphopeptide was determined by solid-phase Edman degradation of the peptide coupled with a Sequelon arylamine membrane (Milligen, Bedford, MA, U.S.A.) using an Applied Biosystems 476A sequenator as described previously [27].

Alternatively, the fractions from the HPLC separation of the tryptic digest of FLAG–IRS-1/PKCδ were purified on a Proxeon $C_{18}$ StageTip (Odense, Denmark) in 0.1 % formic acid/water and eluted with 5 μl of 50 % (v/v) methanol/0.5 % formic acid. The samples were analysed by nanoelectrospray ionization MS on a Q-TOF2 (Waters, Manchester, U.K.) mass spectrometer and the parent ions were subjected to collision-induced dissociation, using operator-defined collision voltages. The fragment ion data were searched using the Mascot algorithm (Matrix Science, London, U.K.) and the sites of phosphorylation determined from the search results were confirmed by manual inspection of the MS/MS spectra.

### Cell culture and treatments

H4IIE FLAG–IRS-1 cells were maintained in Dulbecco's modified Eagle's medium containing 5 % (v/v) foetal bovine serum and 5 % (v/v) foetal calf serum at 37 °C and 5 % $CO_2$, then serum-deprived for 10–18 h in Dulbecco's modified Eagle's medium containing 0.2 % BSA. CHO/IR cells (Chinese-hamster ovary cells overexpressing the insulin receptor) [28] were maintained in Hams F-12 containing 10 % foetal bovine serum at 37 °C and 5 % $CO_2$. CHO/IR cells, approx. 80 % confluent, were transfected in complete growth media with FuGENE 6 (Roche, Indianapolis, IN, U.S.A.) and 1 μg (6-well plate) or 5 μg (10 cm dish) of pcDNA 3.1(+) zeo FLAG–IRS-1 and pcDNA 3 neo PKCδ or pcDNA 3 neo CA-PKCδ (where CA stands for constitutively active) or the vectors lacking an insert. At 12 h post-transfection, cells were serum-deprived for 12 h in Ham's F-12 containing 0.2 % BSA. H4IIE FLAG–IRS-1 and CHO/IR cells were pretreated with PMA or DMSO for 30 min before insulin stimulation.

### Immunoprecipitation, immunoblotting and Far-Western immunoblotting

H4IIE cells were lysed by shaking on ice for 20 min with 50 mM Hepes (pH 7.5), 150 mM NaCl, 1 % Nonidet P40, 1 mM $NA_3VO_4$, 100 nM okadaic acid and 1× protease inhibitor set I cocktail (Calbiochem). Cellular debris was removed by centrifugation at 16 000 ***g*** for 15 min at 4 °C. The protein content was determined by the bicinchoninic acid assay. Approx. 20–30 and 200–300 μg of proteins were used for analyses of total cell lysates and immunoprecipitations respectively. Immunoprecipitations were performed with 30 μl of anti-FLAG agarose at 4 °C for 3 h. Immunoprecipitated IRS-1 proteins were washed with 20 mM Tris (pH 7.4), 200 mM NaCl and 0.1 % Triton X-100, then with 50 mM Tris (pH 7.4) and 500 mM NaCl, next with 50 mM Tris (pH 7.4) and 150 mM NaCl, and finally with 20 mM Tris (pH 7.4) and 200 mM NaCl. Agarose beads were resuspended in 1× sample buffer [58 mM Tris, pH 6.8/1 % SDS/40 % (v/v) glycerol/0.1 M DTT]. Samples were boiled for 5 min and subjected to SDS/PAGE using 6, 7.5 or 10 % (v/v) Tris/glycine gels, then transferred on to nitrocellulose (Schleicher and Schuell, Keene, NH, U.S.A.). Membranes were incubated overnight with various antibodies at 4 °C. The membranes were washed, incubated with horseradish peroxidase-conjugated secondary antibody (1:5000) for 1 h at room temperature and washed again. Detection was then performed with an enhanced chemiluminescent substrate.

Far-Western blotting with PI3K was performed as described previously [16]. Briefly, GST–p85-2SH2 (2.5 μg) was incubated with polyclonal anti-GST (10 μg) in 200 μl of binding buffer [PBS/0.5 % Tween 20/2 % (w/v) BSA] for 1 h at room temperature. The protein–antibody complex was then added to membranes in 10 ml of binding buffer for 3 h at room temperature. Detection was performed as described above.

### Statistical analysis

Chemiluminescent signals were directly quantified using the Kodak 440CF Image Station and Kodak 1D v.3.5.3 software. The absolute integration value of the immunoreactive bands

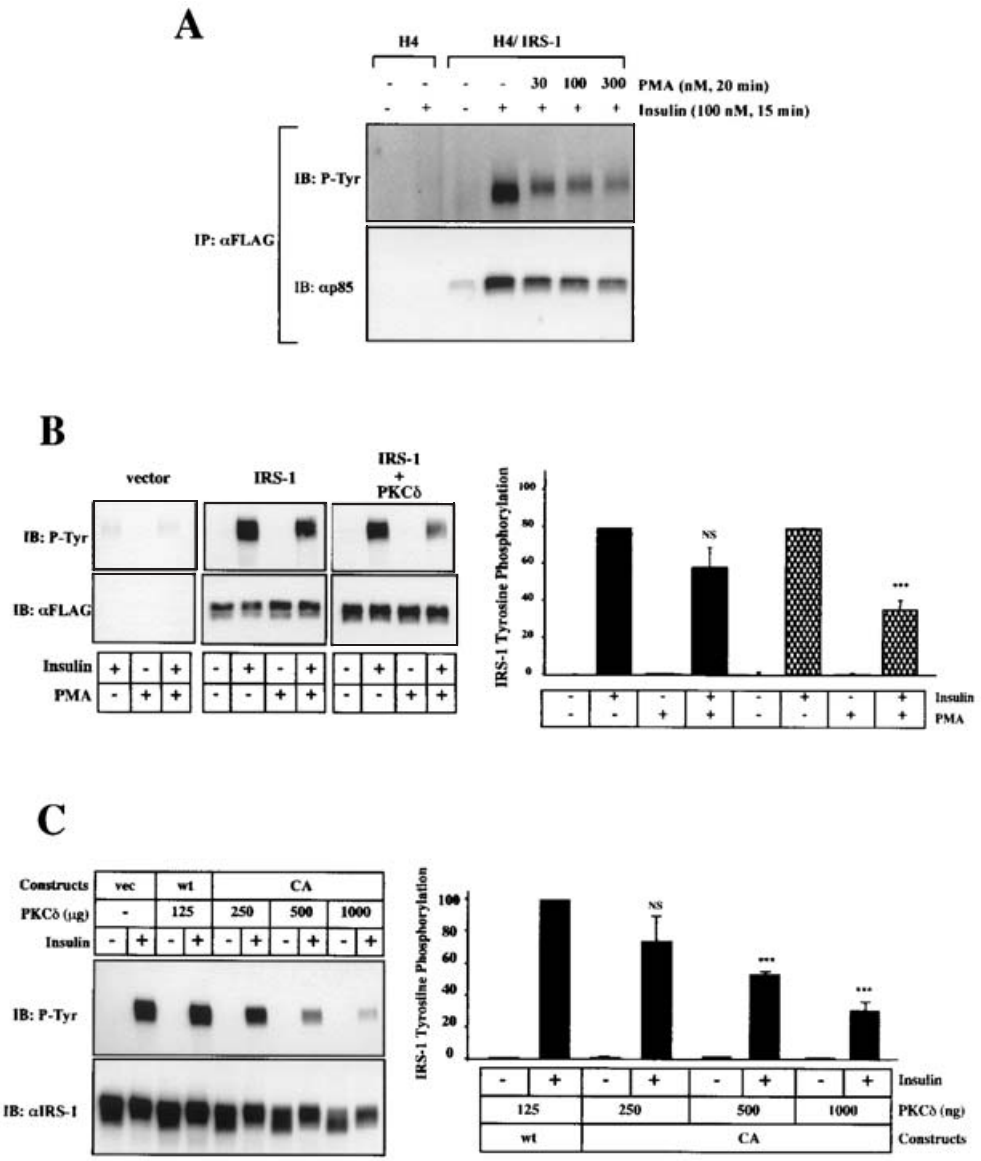


**Figure 1 PKCδ-mediated inhibition of insulin-stimulated IRS-1 tyrosine phosphorylation and PI3K association in cell-culture models**

(**A**) H4IIE cells stably expressing a FLAG-tagged wild-type human IRS-1 (H4 wt IRS-1) were pretreated with the indicated amounts of PMA and then stimulated with insulin and lysed. Anti-FLAG immunoprecipitates from these lysates were separated by SDS/PAGE (6 % gel), transferred on to nitrocellulose and blotted with anti-IRS-1 or anti-p85 antibodies. Representative blots from two independent experiments are shown. IP, immunoprecipitation. (**B**) CHO/IR cells were co-transfected with pcDNA3.1(+) (vector), FLAG–IRS-1 (IRS-1) or wild-type PKCδ (PKCδ) as described in the Experimental section. Cells were serum-deprived for 12 h, pretreated with PMA and then stimulated with insulin. Tyrosine phosphorylation was determined by blotting with 4G10 antibodies (P-Tyr). The total amount of expressed IRS-1 was determined by immunoblotting (IB) with anti-FLAG antibodies. A representative blot is shown (left panel). Results were quantified and are expressed as the amount of tyrosine-phosphorylated IRS-1 normalized for the amount of total IRS-1 present; results are the means ± S.E.M. for three independent experiments (right panel). Solid bar, IRS-1; speckled bar, IRS-1 + PKCδ. NS, not significant, $P > 0.05$; ***$P < 0.005$ versus non-treated. (**C**) CHO/IR cells were co-transfected with FLAG–IRS-1 (IRS-1) and pcDNA3.1(−) (vector), wild-type PKCδ (wt) or constitutively active PKCδ (CA) as described in the Experimental section. Cells were serum-deprived for 12 h and then stimulated with insulin. Tyrosine phosphorylation was determined as described above. A representative blot is shown (left panel). Results have been normalized for the amount of total IRS-1 present and are the means ± S.E.M. for three independent experiments (right panel). NS, not significant, $P > 0.05$; ***$P < 0.005$ versus wt.

minus background was determined. Statistical significance was determined by Student's $t$ test ($\alpha = 0.05$).

## RESULTS

### Activation of PKCδ inhibits IRS-1 function

To determine the extent of inhibition of insulin signalling by endogenous PKC in our system, H4IIE cells expressing a FLAG-tagged wild-type human IRS-1 (H4 wt IRS-1 cells) [29] were treated with various amounts of PMA for 20 min and then stimulated or unstimulated with insulin for 15 min. IRS-1 immunoprecipitates were analysed for tyrosine phosphorylation and the amount of associated PI3K. A dose-dependent decrease in insulin-stimulated IRS-1 tyrosine phosphorylation and PI3K association was detected in H4 wt IRS-1 cells pretreated with PMA (Figure 1A). Since PMA activates both cPKC and nPKC isoenzymes in H4IIE cells ([30] and results not shown), we sought to determine if one of the nPKCs, PKCδ, could inhibit IRS-1 function. CHO/IR cells were transiently co-transfected with FLAG-tagged human

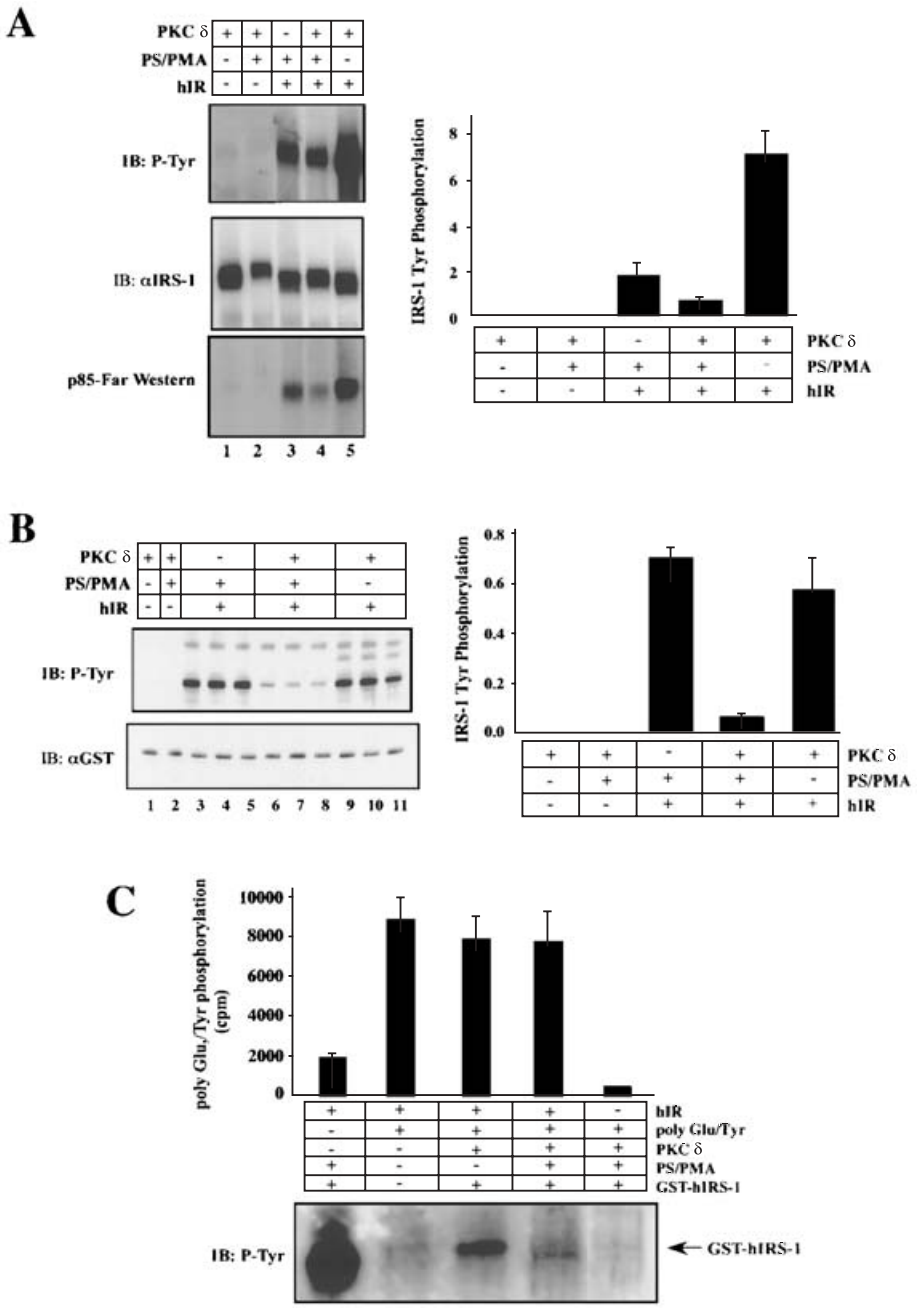


**Figure 2 Inhibition of human insulin-receptor-kinase-catalysed IRS-1 tyrosine phosphorylation by PKCδ phosphorylation of recombinant IRS-1 proteins**

Recombinant FLAG–IRS-1 (**A**) or GST–hIRS-1 (**B**) was incubated in the absence or presence of 100 ng of recombinant PKCδ expressed and purified from Sf9 cells and 100 ng/ml PS and PMA (PS/PMA) to activate PKCδ as described in the Experimental section. After precipitation and washing, IRS-1 was incubated in the absence or presence of activated hIR. Tyrosine phosphorylation was determined by blotting with PY20 antibodies (P-Tyr). PI3K binding was assessed using a GST–p85 SH2 domain recombinant protein (p85-Far Western) as described in the Experimental section. Total amount of IRS-1 was determined by immunoblotting with anti-FLAG (**A**) or anti-GST (**B**) antibodies. A representative immunoblot is shown (left panel). Tyrosine phosphorylation results have been normalized for the amount of total IRS-1 present and are the means ± S.E.M. for three independent experiments (right panel). (**C**) GST–hIRS-$1^{288-678}$ was incubated in the absence or presence of 100 ng of recombinant PKCδ and PS/PMA to activate PKCδ as described above. After precipitation and washing, GST–hIRS-$1^{288-678}$ was incubated with 3 μCi of [$\gamma$-$^{32}$P]ATP in the absence or presence of activated hIR and the exogenous substrate poly(Glu/Tyr) (1 mg/ml). The amount of radioactivity incorporated into the poly(Glu/Tyr) was quantified and expressed as the means ± S.E.M. for three independent experiments (upper panel). Tyrosine phosphorylation was determined as described above and is shown as a representative immunoblot (lower panel).

IRS-1 (FLAG–IRS-1) without or with PKCδ. Cells were treated with 50 nM PMA for 20 min to activate PKCδ and then stimulated or not stimulated with insulin for 10 min. Cell lysates were analysed for IRS-1 tyrosine phosphorylation and normalized to the amount of IRS-1 as assessed by immunoblotting with anti-FLAG antibodies. A 20 ± 9 % decrease in insulin-stimulated IRS-1 tyrosine phosphorylation was detected in CHO/IR cells transfected with only IRS-1 and pretreated with PMA (Figure 1B). However, when IRS-1 was co-transfected with PKCδ, a greater degree of inhibition (50 ± 9 %) of insulin-stimulated IRS-1 tyrosine phosphorylation was observed (Figure 1B). Since PMA may have cellular targets other than PKC isoenzymes, we sought

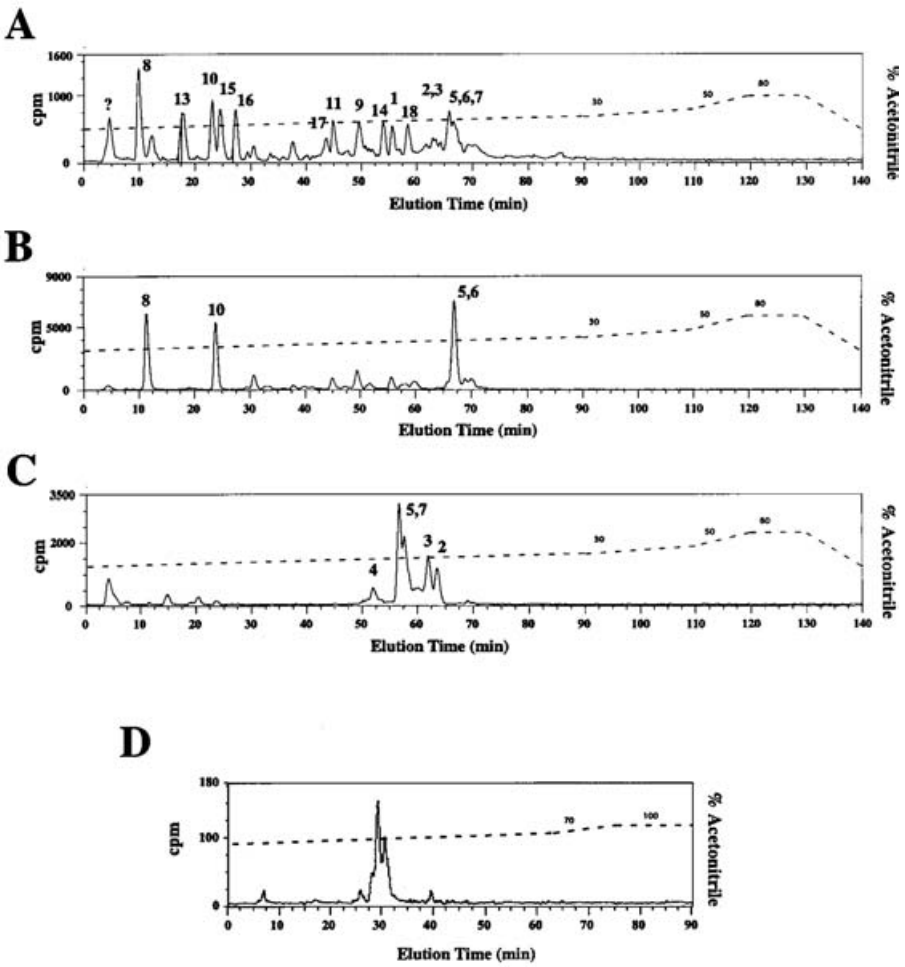


**Figure 3 HPLC analyses of PKC$\delta$-phosphorylated recombinant IRS-1 phosphopeptides**

PKC$\delta$-phosphorylated IRS-1 was separated by SDS/PAGE and then digested with trypsin as described in the Experimental section. The resulting $^{32}$P-labelled peptides were chromatographed on a Vydac 218TP54 $C_{18}$ column (Separations Group, Hesperia, CA, U.S.A.) equilibrated in 0.1 % (v/v) trifluoroacetic acid in water. The column was developed with a linear acetonitrile gradient (broken line) at a flow rate of 0.8 ml/min, and fractions of 0.4 ml were collected; $>$ 80 % of the radioactivity applied to the column was recovered in the fractions. Similar profiles were obtained in three separate experiments. The positions of the phosphopeptides termed 1–18 are indicated. (**A**) Recombinant full-length FLAG–IRS-1 phosphorylated with PKC$\delta$ and digested with trypsin. (**B**) Recombinant GST–hIRS-1$^{288-678}$ phosphorylated with PKC$\delta$ and digested with trypsin. (**C**) Recombinant GST–PH-PTB-rIRS-1$^{1-309}$ phosphorylated with PKC$\delta$ and digested with trypsin. (**D**) Recombinant GST–PH-PTB-rIRS-1$^{1-309}$ phosphorylated with PKC$\delta$ and digested with Lys-C.

to confirm the above results using a constitutively active mutant of PKC$\delta$ [31]. CHO/IR cells were transiently co-transfected with FLAG–IRS-1 and either the wild-type or constitutively active PKC$\delta$ mutant. Cell lysates were analysed for total and tyrosine-phosphorylated IRS-1 as described above. A decrease in total IRS-1 levels was observed with higher amounts of constitutively active PKC$\delta$ mutant, possibly owing to an enhanced degradation of this molecule. In addition, a decrease in insulin-stimulated IRS-1 tyrosine phosphorylation per IRS-1 molecule was detected in CHO/IR cells transfected with both FLAG–IRS-1 and the constitutively active PKC$\delta$ mutant (Figure 1C).

### PKC$\delta$ inhibits insulin-receptor-kinase-catalysed IRS-1 tyrosine phosphorylation *in vitro*

IRS-1 is an *in vitro* substrate for a number of Ser/Thr kinases [32–38]. As a first step to determine if PKC$\delta$ plays a direct role in the inhibition of insulin signalling through IRS-1, recombinant PKC$\delta$ was tested for its ability to phosphorylate and inhibit the subsequent insulin receptor tyrosine phosphorylation of recombinant full-length human IRS-1 and a human IRS-1–GST-fusion protein (GST–hIRS-1$^{288-678}$). Preincubation of IRS-1 with activated PKC$\delta$ *in vitro* inhibited the insulin-receptor-kinase-catalysed IRS-1 tyrosine phosphorylation by approx. 90 % as assessed by immunoblotting (Figure 2A, lane 4 versus lane 5 and Figure 2B, lanes 6–8 versus lanes 9–11). Similar results were obtained when the insulin-receptor-kinase-catalysed IRS-1 tyrosine phosphorylation was assessed using a [$\gamma$-$^{32}$P]ATP kinase assay (results not shown). We have also detected a decrease in insulin-receptor-kinase-catalysed IRS-1 tyrosine phosphorylation using recombinant full-length human IRS-1 when PS and PMA are present and PKC$\delta$ is absent (Figure 2A, lane 3 versus lane 4). The functional relevance of PKC$\delta$-mediated inhibition of the insulin-receptor-kinase-catalysed IRS-1 tyrosine phosphorylation was assessed in a PI3K far-Western immunoblot, using a GST–p85 subunit recombinant protein [16]. Consistent with the ability of PKC$\delta$ to modulate the overall IRS-1 tyrosine phosphorylation, PKC$\delta$ inhibited the association of the p85 subunit of PI3K with tyrosine-phosphorylated IRS-1 (Figure 2A, bottom panel, lane 4 versus lane 5).

**Table 1 Characterization of HPLC-purified phosphopeptides from a tryptic digest of *in vitro* PKC$\delta$-phosphorylated IRS-1**

IRS-1 was phosphorylated and the phosphopeptides generated were purified on reversed-phase HPLC as described in the Experimental section. Seventeen major peaks of radioactivity were detected (1–18; Figure 3). Phosphopeptides were analysed by solid-phase sequencing and MALDI–TOF-MS or by nanoelectrospray ionization MS. Peptide masses [M + H] are expressed as monoisotopic mass or average mass (av), except for multiply charged ions detected by electrospray ionization MS. For each phosphopeptide, the peptide number, amino acid sequence (with the site of phosphorylation underlined; 'm', methionine sulphone) and the amino acid sequence number of human IRS-1 are shown. In columns 4 and 5 where peptide masses are given, the theoretical peptide mass (Expected) is shown first, followed by the mass determined by MS (Found).

| | | | Mass ($m/z$) | |
|---|---|---|---|---|
| Peptide no. | Amino acid sequence | Site | Expected | Found |
| 1 | AmSDEFRPR* | Ser-261 | $602.743^{2+}$ | $602.745^{2+}$ |
| 2 | SKSQSSSNCSNPISVPLR* | Ser-270 | $692.653^{3+}$ | $692.693^{3+}$ |
| 3 | SQSSSNCSNPISVPLR† | Ser-272 | 1860.832 | 1860.830 |
| 4 | HHLNNPPPSQVGLTRR | Ser-295 or Thr-300 | 1902.945 | 1902.966 |
| 5 | TESITATSPPASmVGGKPGSFR | Ser-307, Ser-323 | 2258.314 (av) | 2258.199 (av) |
| 6 | SRTESITATSPPASmVGGKPGSFR† | Ser-307, Ser-323 | 2501.581 (av) | 2502.234 (av) |
| 7 | SRTESITATSPPASmVGGKPGSFR†‡ | Ser-303, Ser-307, Ser-323 | 2581.561 (av) | 2581.078 (av) |
| 8 | HRGSAR† | Ser-362 | 763.336 | 763.341 |
| 9 | SIPMPASR* | Ser-374 | $477.702^{2+}$ | $477.706^{2+}$ |
| 10 | SSFR† | Ser-441 | 576.218 | 576.151 |
| 11 | HSAFVPTR* | Ser-574 | $497.716^{2+}$ | $497.726^{2+}$ |
| 12 | LPGHRHSAFVPTR† | Ser-574 | 1554.769 | 1554.734 |
| 13 | SFK | Ser-770 | 461.170 | 461.180 |
| 14 | HQHLRLSTSSGR‡ | Ser-791 | 1458.697 | 1458.681 |
| 15 | LSTSSGR | Ser-794 | 787.335 | 787.337 |
| 16 | KVDTAAQTNSR* | Thr-847 | $635.774^{2+}$ | $635.789^{2+}$ |
| 17 | CPSQLQPAPR | Ser-924 | 1281.581 | 1281.567 |
| 18 | LGPAPPGAASICRPTR†‡ | Ser-973 | 1748.867 | 1748.853 |

* Phosphopeptides and phosphorylation site(s) determined using nanoelectrospray ionization MS.
† Phosphopeptides and phosphorylation site(s) determined in at least two independent phosphorylations.
‡ Phosphopeptides and phosphorylation site(s) from a tryptic digest of *in vitro* PKC$\theta$-phosphorylated IRS-1.

These results suggest that PKC$\delta$ inhibits the ability of the insulin receptor kinase to tyrosine-phosphorylate IRS-1. However, this could occur in two ways: either PKC$\delta$ could be phosphorylating IRS-1 and modifying its ability to be phosphorylated via the insulin receptor or it could be that PKC$\delta$ is phosphorylating the insulin receptor and modifying its enzymic activity. This latter possibility is supported by reports that PKC$\delta$ can associate with and phosphorylate the insulin receptor, although the functional consequence on intrinsic insulin receptor kinase activity has not been consistently observed [12,39]. To determine if PKC$\delta$ directly affects the tyrosine kinase activity of the insulin receptor under the present conditions, an experiment similar to the experiment using GST–hIRS-$1^{288-678}$-fusion protein shown in Figure 2(B) was performed, except that an exogenous substrate [poly(Glu/Tyr)] and [$\gamma$-$^{32}$P]ATP were added along with the insulin receptor kinase. No difference was observed in the phosphorylation of the exogenous substrate under conditions where the tyrosine phosphorylation of the GST–hIRS-$1^{268-699}$-fusion protein was inhibited (Figure 2C, lane 3 versus lane 4). These results are consistent with the hypothesis that it is the phosphorylation of IRS-1 by PKC$\delta$ which is inhibitory towards insulin-receptor-kinase-catalysed IRS-1 tyrosine phosphorylation.

### nPKC isoenzymes phosphorylate IRS-1 on multiple sites

To confirm that IRS-1 is a substrate for PKC$\delta$ and to determine the sites of phosphorylation, recombinant full-length human IRS-1 was phosphorylated with recombinant PKC$\delta$ and the radiolabelled IRS-1 was digested in gel with trypsin. Radiolabelled phosphopeptides (approx. 85–90%) were released from the gel (results not shown). The peptides were separated by HPLC and a complex phosphopeptide map was observed (Figure 3A). Two additional recombinant proteins were used to confirm and further localize PKC$\delta$ phosphorylation sites in IRS-1. GST–hIRS-$1^{288-678}$ (Figure 3B) and GST–PH-PTB-rIRS-$1^{1-309}$ (Figure 3C) were phosphorylated with recombinant PKC$\delta$ and analysed as described above. Further analyses of GST–PH-PTB-rIRS-$1^{1-309}$ revealed that all of the PKC$\delta$ phosphorylation sites in this recombinant protein were found within a single Lys-C peptide amino acids 250–303 (Figure 3D). In total, 18 phosphorylation sites were identified as described in the Experimental section (Table 1). Eight of these sites (Ser-272, Ser-303, Ser-307, Ser-323, Ser-362, Ser-441, Ser-574 and Ser-973) were found in at least two independent experiments (Table 1). Solid-phase sequencing of the phosphopeptides confirmed the identified PKC$\delta$ phosphorylation sites (Figure 4). Half the identified PKC$\delta$ phosphorylation sites in IRS-1 were clustered close to the PTB domain between amino acids 261 and 362 (Figure 5).

It is of interest to note that an *in silico* PKC$\delta$ phosphorylation of IRS-1 at low stringency with ScanSite v2.0 predicts a total of 17 phosphorylation sites [40]. However, our results indicate that only three of the predicted sites (Ser-272, Ser-307 and Ser-441) are phosphorylated by PKC$\delta$. Thus most of the PKC$\delta$ phosphorylation sites in IRS-1 were not predicted with ScanSite. The inability of ScanSite to predict accurately tyrosine phosphorylation sites has also been reported previously [41].

PKC$\delta$ and PKC$\theta$ form a subgroup of nPKC isoenzymes based on amino acid sequence homology [7]. These isoenzymes are 76 and 82% identical in the kinase- and phorbol ester-binding (C1) domains respectively. On the basis of the sequence homology between PKC$\delta$ and PKC$\theta$, it is plausible that IRS-1 is a substrate for PKC$\theta$. Not surprisingly, IRS-1 is phosphorylated *in vitro* to a similar extent by both PKC$\theta$ and PKC$\delta$ (results not shown). To determine the sites of phosphorylation, recombinant full-length human IRS-1 was phosphorylated with recombinant PKC$\theta$ and analysed as described above. Four phosphopeptides containing the following phosphorylation sites were identified: Ser-303, Ser-307, Ser-323, Ser-574 and Ser-973 (Table 1 and results not shown).

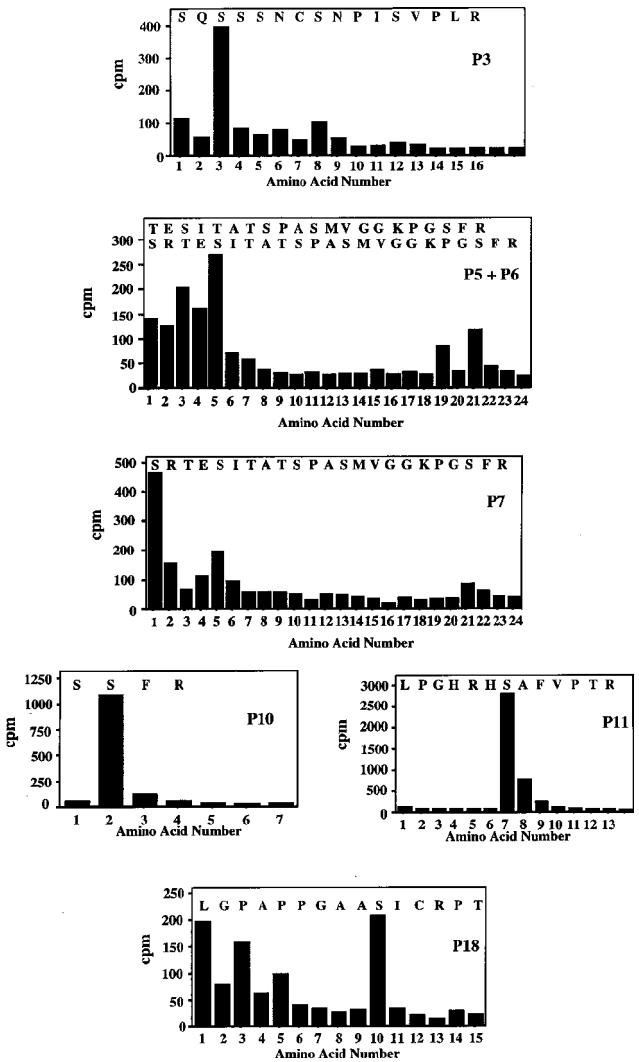


**Figure 4 Solid-phase sequence analysis of PKCδ-phosphorylated recombinant IRS-1 phosphopeptides**

Aliquots of the major $^{32}$P-labelled peptides derived from phosphorylated IRS-1 (Figure 3) were covalently coupled with a Sequelon arylamine membrane and analysed on an Applied Biosystems 476A sequenator. $^{32}$P radioactivity was measured after each cycle of Edman degradation. By database searching against predicted IRS-1 tryptic peptides and phospho-amino-acid analysis, in combination with MALDI–TOF-MS, the sites of phosphorylation in each of the peptides were identified (Table 1). The putative amino acid sequence of each peptide, deduced from a combination of MS and solid-phase sequencing, is indicated on the top of each panel. Phosphopeptide peaks P1–P6 are the same as in Figure 3.

Previous studies have indicated that purified, recombinant IRS-1 has an associated Ser/Thr kinase [42], which is also observed in IRS-1 immunoprecipitated from a variety of cultured cells (results not shown). When recombinant full-length human IRS-1 was incubated in the kinase reaction buffer in the absence of added PKCδ or PKCθ and analysed as described above, a single phosphopeptide was detected. The site of phosphorylation was determined to be Ser-791 (Table 1 and results not shown).

### *In vitro* analysis of PKCδ phosphorylation site mutants in IRS-1

One of the major PKCδ phosphorylation sites in IRS-1 identified above was Ser-307. This site is five residues away from Ser-312, a major desensitization site in IRS-1 that has been shown to have a dual mechanism of modulating insulin action: uncoupling the interaction of IRS-1 with the insulin receptor, as well as the targeting of IRS-1 to the degradation pathway [29,43]. To determine if the Ser-307 PKCδ phosphorylation site was contributing to the *in vitro* inhibition of IRS-1 tyrosine phosphorylation, IRS-1 Ser-307 (analogous to Ser-302 in rat IRS-1) was replaced by an alanine residue. Phosphorylation of the GST–hIRS-1$^{288-678}$ Ser-307 mutant was decreased by 50–60% in an *in vitro* PKCδ kinase assay (Figure 6A). Furthermore, mutation of Ser-307 in GST–hIRS-1$^{288-678}$ partially abrogated the PKCδ effect on insulin-receptor-kinase-catalysed IRS-1 tyrosine phosphorylation (Figure 6B). Mutagenesis of the other major PKCδ phosphorylation sites found within the IRS-1-fusion protein (Ser-323 and Ser-574) resulted in a 75–80% decrease in *in vitro* phosphorylation by PKCδ (Figure 7A). More importantly, the GST–hIRS-1$^{288-678}$ Ser-307/Ser-323/Ser-574 triple mutant fully abrogated the effect of PKCδ on insulin-receptor-kinase-catalysed IRS-1 tyrosine phosphorylation (Figure 7B).

## DISCUSSION

Insulin resistance both in cultured cells and *in vivo* is associated with hyperphosphorylation of IRS-1 on Ser/Thr residues [6,44]. Counter-regulatory hormonal activation, pro-inflammatory cytokine production/cellular stress and inhibition of protein phosphatases 1 and 2A all result in the hyper-Ser/Thr phosphorylation of IRS-1 [6,44]. A unifying consequence of IRS-1 hyper-Ser/Thr phosphorylation is the negative modulation of insulin-stimulated IRS-1 tyrosine phosphorylation. Potential mechanisms by which IRS-1 tyrosine phosphorylation is decreased include the following: (i) uncoupling of IRS proteins from cytoskeletal/membrane components, which thereby causes translocation of IRS-1 away from the insulin receptor at the plasma membrane to the cytosol [45]; (ii) inhibition of the insulin receptor–IRS-1

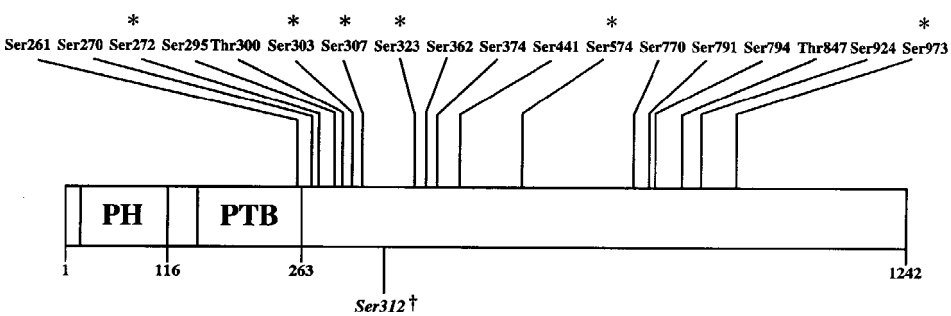


**Figure 5 Schematic diagram of human IRS-1 PKCδ phosphorylation sites**

Amino acid residues from 1 to 1242 are indicated. The N-terminal PH and PTB domains are represented as boxes. All 18 PKCδ phosphorylation sites with their relative location in IRS-1 are indicated. PKCδ phosphorylation sites identified in at least two independent experiments are marked with asterisks. †, Previously identified c-Jun N-terminal kinase phosphorylation site.

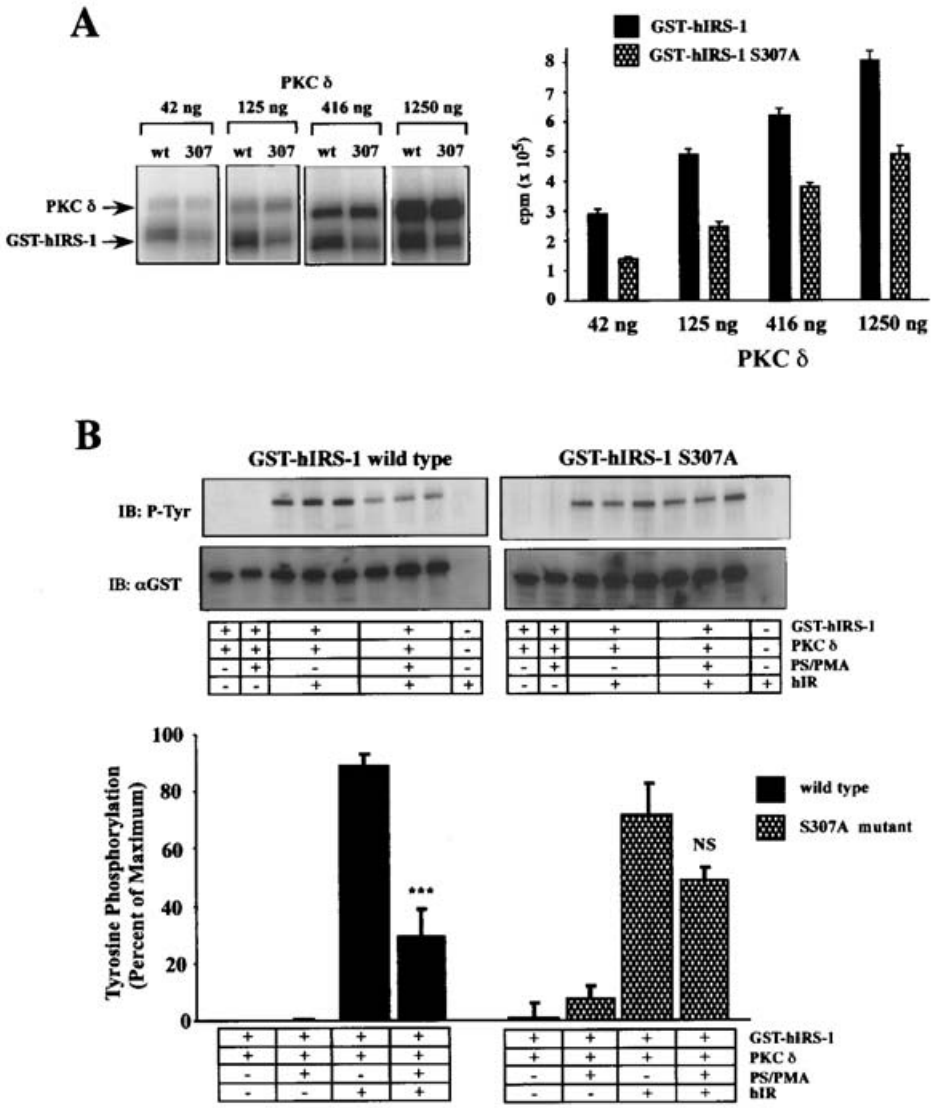


**Figure 6 PKCδ phosphorylation of a recombinant IRS-1 S307A (Ser$^{307}$ → Ala) mutant protein and partial abrogation of the inhibitory effect of PKCδ on human insulin-receptor-kinase-catalysed IRS-1 tyrosine phosphorylation**

(**A**) IRS-1 Ser-307 was mutated to Ala as described in the Experimental section. Recombinant wild-type GST–hIRS-1$^{288-678}$ (wt) and the Ser-307 mutant GST–hIRS-1$^{288-678}$ (307) were incubated in the presence of various amounts of recombinant PKCδ and 100 ng/ml PS and PMA (PS/PMA) and in the presence of [$\gamma$-$^{32}$P]ATP as described in the Experimental section. A representative autoradiogram is shown (left panel) and quantification of the IRS-1 phosphorylation is shown as the means ± S.E.M. for three independent experiments (right panel). (**B**) Recombinant wild-type GST–hIRS-1$^{288-678}$ and the Ser-307 mutant GST–hIRS-1$^{288-678}$ were incubated in the absence or presence of 832 ng of recombinant (from *Escherichia coli*) PKCδ and 100 ng/ml PS and PMA (PS/PMA) as described in the Experimental section. After precipitation and washing, IRS-1 was incubated in the absence or presence of activated hIR. Tyrosine phosphorylation and the total amount of IRS-1 were determined as described in the legend to Figure 2. Representative immunoblots are shown (upper panels) and quantification of the tyrosine-phosphorylated IRS-1 is shown as the means ± S.E.M. for two independent experiments (lower panels). NS, not significant, $P > 0.05$; ***$P < 0.005$ versus non-activated PKCδ.

interaction [46]; (iii) proteosome-mediated IRS-1 degradation [29,47]; and (iv) inhibition of the insulin receptor phosphotransferase activity [33].

Since increases in the level of NEFAs (non-esterified fatty acids) are common in insulin-resistant states such as obesity and Type II diabetes, recent studies by a number of investigators have examined the mechanism(s) of insulin resistance after NEFA infusion or after dietary lipid overabundance [6,17,20–23,48]. These studies have implicated the nPKCs, either PKCδ, PKCθ or PKCε, in lipid-induced insulin resistance in humans and rats. Consistent with these studies is the observation that nPKCs are activated in muscles from hyperinsulinaemic and hyperglycaemic humans and rats [49,50].

Therefore, in the present study, we have focused on the mechanism(s) of one of the nPKCs, PKCδ, in mediating insulin desensitization. We have found that the PKCδ inhibition of insulin-receptor-kinase-catalysed IRS-1 tyrosine phosphorylation can occur as a consequence of the direct Ser/Thr phosphorylation of IRS-1 by PKCδ. First, we demonstrate that recombinantly produced PKCδ (expressed and purified from either insect cells or bacteria) can directly phosphorylate, *in vitro*, three different recombinant forms of IRS-1. The phosphorylation of IRS-1 by PKCδ is quite complex, since we were able to identify 18 specific PKCδ phosphorylation sites in IRS-1. Eight of these sites were found in at least two independent experiments and at least six of these sites are common to both PKCδ and PKCθ. Secondly,

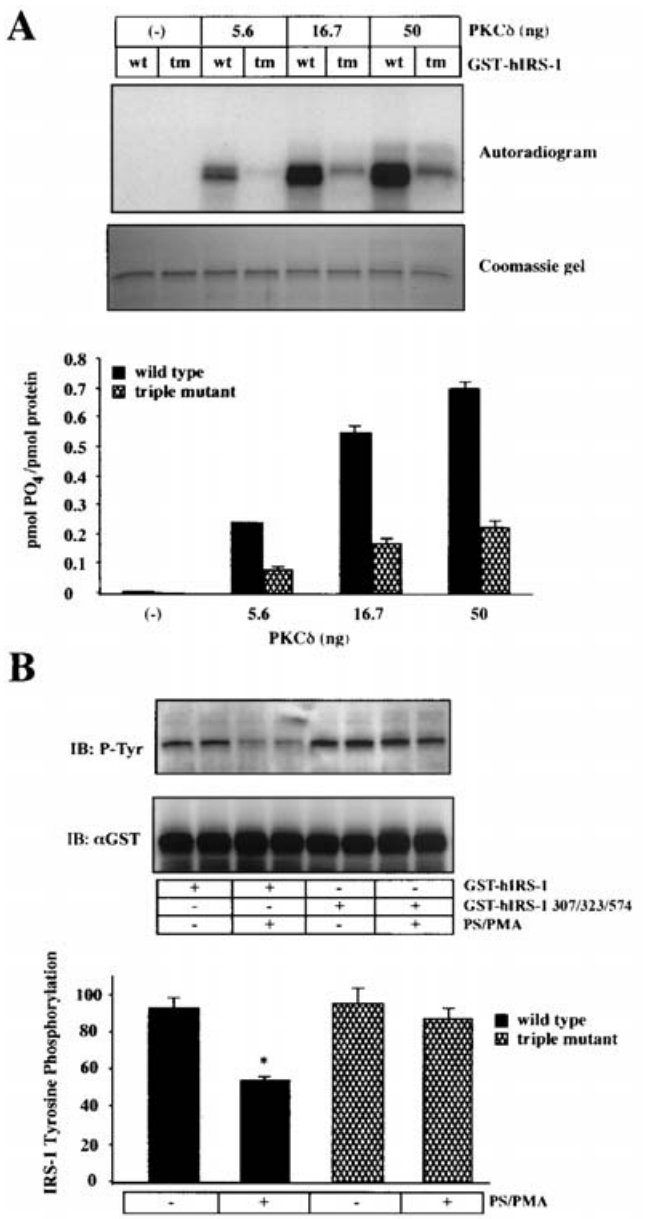


**Figure 7 PKC$\delta$ phosphorylation of a recombinant IRS-1 S307A, S323A and S574A mutant protein and complete abrogation of the inhibitory effect of PKC$\delta$ on human insulin-receptor-kinase-catalysed IRS-1 tyrosine phosphorylation *in vitro***

(**A**) IRS-1 Ser-323 and Ser-574 were mutated to Ala using the Ala-307 mutant as a template as described in the Experimental section. Recombinant wild-type (wt) and Ser-307/Ser-323/Ser-574 mutant (tm) GST–hIRS-$1^{288-678}$ were incubated in the presence of the indicated amounts of recombinant (baculovirus) PKC$\delta$ and 100 ng/ml PS and PMA (PS/PMA) and [$\gamma$-$^{32}$P]ATP as described in the Experimental section. The amount of phosphorylated IRS-1 is shown as a representative autoradiogram (upper panel) and, after quantification, as the means $\pm$ S.E.M. for three independent experiments (lower panel). (**B**) Recombinant wild-type GST–hIRS-$1^{288-678}$ and the Ser-307/Ser-323/Ser-574 mutant GST–hIRS-$1^{288-678}$ were incubated in the absence or presence of 50 ng of recombinant (baculovirus) PKC$\delta$ and 100 ng/ml PS and PMA (PS/PMA) as described in the Experimental section. After precipitation and washing, IRS-1 was incubated with activated hIR. The amounts of tyrosine phosphorylation and total IRS-1 were quantified as described in the legend to Figure 2 and are shown as a representative immunoblot (upper panel) and as the means $\pm$ S.E.M. for two independent experiments (lower panel). ***$P < 0.05$ versus non-activated PKC$\delta$.

we were able to demonstrate that the *in vitro* phosphorylation of IRS-1 by PKC$\delta$ resulted in a decreased ability of the insulin receptor to tyrosine-phosphorylate this molecule. In contrast, under the same conditions, no decrease in the ability of the insulin receptor to phosphorylate a distinct exogenous substrate was observed. Finally, a mutant IRS-1 in which three serine phosphorylation sites were replaced by alanine residues completely abrogated the effect of PKC$\delta$ on insulin-receptor-kinase-catalysed IRS-1 tyrosine phosphorylation. These results indicate that the direct phosphorylation of IRS-1 by PKC$\delta$ is sufficient to inhibit the ability of the insulin receptor to tyrosine-phosphorylate the IRS-1 molecule.

However, the *in vivo* role of direct phosphorylation of IRS-1 by PKC$\delta$ as well as other PKCs is less clear. In a cell, PKCs can activate additional downstream kinases, which may also phosphorylate IRS-1. Given that IRS-1 contains 182 serine and 60 threonine residues in over 40 consensus Ser/Thr phosphorylation sites, the potential complexity of *in vivo* phosphorylation of IRS-1 is evident. For example, cPKC-mediated activation of MAPK was found to result in the phosphorylation of IRS-1 Ser-616 and the modulation of insulin-stimulated PI3K activity [16,33]. It has also been suggested that activation of an nPKC by lipid infusion leads to the phosphorylation of IRS-1 on Ser-312 [22], a site phosphorylated by c-Jun N-terminal kinase and other Ser/Thr kinases [29,32] but not by PKC$\delta$ and PKC$\theta$ (the present study). Thus our results suggest that nPKC may also have an indirect effect on IRS-1 Ser/Thr phosphorylation.

We have also identified a novel IRS-1 serine phosphorylation site (Ser-791) that is phosphorylated by an unknown associated kinase. The relevance of phosphorylation at this site is not known. It will be of interest to determine if phosphorylation at this site has a positive or negative regulatory role [2,42].

The high number of direct and indirect phosphorylation sites in IRS-1 complicates the functional analysis of these sites *in vivo*. Indeed, although three of the identified PKC$\delta$ phosphorylation sites (Ser-307, Ser-323 and Ser-574) appeared to comprise a major portion of the *in vitro* phosphorylations (as demonstrated by a 75–80 % decrease in phosphate incorporation into this mutant), as well as being responsible for a major portion of the inhibitory effect on insulin receptor tyrosine phosphorylation *in vitro*, expression of the full-length IRS-1 with these same three mutations did not have a major effect on the *in vivo* inhibitory effect of overexpressed PKC$\delta$ (results not shown). One explanation for these results is that the inhibitory effect of activation of PKC$\delta$ *in vivo* is indirect, i.e. via the activation of another kinase which phosphorylates IRS-1 on additional sites (i.e. Ser-312, as described above). Alternatively, it is possible that in the context of the cell, where IRS-1 protein–protein interactions and subcellular localization play an important role, PKC$\delta$ phosphorylation sites other than Ser-307, Ser-323 and Ser-574 are more important in the desensitization process than those observed *in vitro*. For example, eight PKC$\delta$ phosphorylation sites in IRS-1 are proximal to the PTB domain, which plays an important role in mediating the IRS-1–insulin receptor interaction. Further testing is required to distinguish between these two possibilities.

It is of interest to note that, recently, rat IRS-1 Ser-318 (corresponding to human Ser-323) was identified as a PKC$\zeta$ phosphorylation site by a novel electrospray ionization MS-based technique [15]. We have detected the same PKC$\delta$ and PKC$\theta$ tryptic phosphopeptide as a monophosphopeptide, diphosphopeptide and triphosphopeptide (Table 1 and results not shown), thus illustrating the power of our phosphopeptide mapping strategy. Even though the functional significance of phosphorylation at this site was not reported, it has been shown that PKC$\zeta$ is a negative regulator of IRS-1 function and insulin signalling [13,14]. It is plausible that PKC$\zeta$ and PKC$\delta$ phosphorylate common sites in IRS-1 such as Ser-323, which in turn plays a role in the inhibition of insulin signalling as a physiological negative-feedback control mechanism and/or under pathological conditions.

In summary, results of the present study clearly demonstrate that PKCδ can interfere with insulin signalling by direct phosphorylation of IRS-1. Identification of several of these sites will allow further studies testing whether these residues are phosphorylated in various models of insulin resistance, including lipid infusion when the nPKCs are activated, as well as testing of their roles in mediating insulin resistance in intact cells.

We are grateful to Dr Michael Gschwendt for providing the pET28 PKCδ plasmid. We thank Dr Jae-Won Soh for generously providing us with the constitutively active PKCδ plasmid. We thank the Post Genomics and Molecular Interactions Centre (University of Dundee) for access to the MS facilities. This work was supported in part by National Institutes of Health grant no. DK34976 (to R. A. R.).

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Received 30 September 2003/28 October 2003; accepted 29 October 2003
Published as BJ Immediate Publication 29 October 2003, DOI 10.1042/BJ20031493