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Serine residues 994 and 1023/25 are important for insulin receptor kinase inhibition by protein kinase C isoforms $\beta 2$ and θ

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Abstract

Aims/hypothesis. Inhibition of the signalling function of the human insulin receptor (HIR) is one of the principle mechanisms which induce cellular insulin resistance. It is speculated that serine residues in the insulin receptor β-subunit are involved in receptor inhibition either as inhibitory phosphorylation sites or as part of receptor domains which bind inhibitory proteins or tyrosine phosphatases. As reported earlier we prepared 16 serine to alanine point mutations of the HIR and found that serine to alanine mutants HIR-994 and HIR-1023/25 showed increased tyrosine autophosphorylation when expressed in human embryonic kidney (HEK) 293 cells. In this study we examined whether these mutant receptors have a different susceptibility to inhibition by serine kinases or an altered tyrosine kinase activity.
Methods. Tyrosine kinase assay and transfection studies.
Results. In an in vitro kinase assay using IRS-1 as a substrate we could detect a higher intrinsic tyrosine kinase activity of both receptor constructs. Additionally, a higher capacity to phosphorylate the adapter protein Shc in intact cells was seen. To test the inhibition by serine kinases, the receptor constructs were expressed in HEK 293 cells together with IRS-1 and protein kinase C isoforms $\beta 2$ and θ. Phorbol ester stimulation of these cells reduced wild-type receptor autophosphorylation to 58 % or 55 % of the insulin simulated state, respectively. This inhibitory effect was not observed with HIR-994 and HIR-1023/25, although all other tested HIR mutants showed similar inhibition induced by protein kinase C.
Conclusion/interpretation. The data suggest that the HIR-domain which contains the serine residues 994 and 1023/25 is important for the inhibitory effect of protein kinase C isoforms $\beta 2$ and θ on insulin receptor autophosphorylation. [Diabetologia (2000) 43: 443–449]

Keywords Insulin receptor inhibition, tyrosine kinase activity, serine phosphorylation, protein kinase C.

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Abbreviations: ECL, enhanced chemiluminescence; HEK, human embryonic kidney; HIR, human insulin receptor; HIR-wt, HIR-wild type; MAPK, mitogen-activated protein kinase; PDK, 3-phosphoinositide-dependent protein kinase; PI3K, phosphatidylinositol 3′-kinase; PKB, protein kinase B; PKC, protein kinase C; PMSF, phenylmethylsulphonyl fluoride; SHP, src homology protein tyrosine phosphatase; TPA, 12-*O*-tetradecanoylphorbol-13-acetate.

The tyrosine kinase activity of the human insulin receptor (HIR) is essential for its signalling function [1]. Insulin-induced autophosphorylation of the receptor β-subunit at tyrosine residues in the kinase domain and in the juxtamembrane domain of the receptor β-subunit is critical for autoactivation of the receptor and for substrate phosphorylation [2, 3]. After the rapid insulin-stimulated tyrosine phosphorylation of the receptor β-subunit, there is a delayed insulin-stimulated increase of serine and threonine phosphorylation of the receptor β-subunit in intact cells [4–7]. Serine phosphorylation occurs as well on the next level of the insulin signalling chain, i. e. receptor sub-

strates like IRS-1 [8] and beyond the level of direct substrates, for instance at the regulatory subunit of the phosphatidylinositol 3-kinase (PI3K) [9].

The relevance of serine and threonine phosphorylation of the receptor and immediate downstream elements is not understood in detail. One possible function could be that phosphorylation of specific serine and threonine residues is part of negative feedback loops originating from serine/threonine kinases which are further downstream in the insulin signalling chain, including PI3K, mitogen activated protein kinase (MAPK), 3-phosphoinositide-dependent kinase-1 (PDK1), protein kinase B (PKB) and protein kinase C (PKC) isoforms [9, 10–13]. The physiological function of negative feedback loops in the insulin signalling chain could be a termination of insulin signals. There is evidence that activation of serine phosphorylation is involved in several examples of inducible cellular insulin resistance [14, 15]. The description of these feedback loops could offer valuable clues to the understanding of how cellular insulin resistance can be prevented.

To identify serine positions in the insulin receptor which are relevant for a modulation of the signalling function of the receptor, we prepared 16 serine to alanine mutants of the HIR. Serines were systematically exchanged in the kinase domain and the C-terminus of the insulin receptor β-subunit either at known phosphorylation sites (1023/25, 1293/94, 1308/09) [16–19] or positions which are conserved in different tyrosine kinase receptors (962, 994, 1037, 1055, 1074/78, 1168, 1177/78/82, 1202, 1263, 1267) [20]. In addition, we mutated three unrelated serine residues (1192, 1258, 1275). We found earlier that serine to alanine exchanges at positions 994 and 1023/25 are associated with increased autophosphorylation [21] and have speculated that these domains are important for receptor inhibition.

In a previous study we could show that co-transfection of the insulin receptor with the PKC isoforms β1 and β2 caused an inhibition of HIR autophosphorylation on phorbol ester stimulation of the cells but no effects were seen with the other PKC isoforms [22]. More recently, we have observed that transfection of HEK 293 cells with IRS-1 in addition to HIR and PKC isoforms did not only augment the inhibitory effect of PKCβ1 and β2 but enabled other PKC isoforms (α, δ, θ) also to reduce receptor autophosphorylation [23]. As HEK 293 cells contain only low concentrations of endogenous IRS-1 these experiments have suggested that the effects of the PKC isoforms on the insulin receptor are dependent on the presence of IRS-1. Because serines 1023/25 were already described as potential PKC phosphorylation sites [16], the aim of this study was to define whether the increased tyrosine autophosphorylation of HIR-994 and HIR-1023/25 reflect altered HIR tyrosine kinase activity. Furthermore, we investigated whether the increased autophosphorylation of HIR-994 and HIR-1023/25 could reflect a different susceptibility to inhibition by protein kinase C. To test this hypothesis HEK 293 cells were co-transfected with different HIR constructs, IRS-1 and PKC isoforms β2 or θ. We used these two isoforms because in our previous studies [22, 23] they had the strongest inhibitory effect on the insulin receptor. Furthermore, we have shown a reduced tyrosine phosphorylation of IRS-1 for these isoforms, potentially caused by increased serine phosphorylation of IRS-1 [23].

The results presented in this study show that the insulin receptor constructs HIR-994 and HIR-1023/25 have a higher tyrosine kinase activity in HEK 293 cells and that the domain which contains the serine residues 994 and 1023/25 is important for the inhibitory effect of PKC isoforms β2 and θ on insulin receptor autophosphorylation.

Materials and methods

Materials. Cell culture reagents and fetal calf serum (FCS) were purchased from Gibco (Eggenstein, Germany); culture dishes were from Greiner (Frickenhausen, Germany). Porcine insulin, aprotinin, phenylmethylsulphonyl fluoride (PMSF), Na_3VO_4, Triton X-100, and dithiothreitol were from Sigma (Munich, Germany). The reagents for SDS-PAGE and western blotting were obtained from Roth (Karlsruhe, Germany) and Bio-Rad (Munich, Germany). Nitrocellulose was from Schleicher & Schuell (Dassel, Germany). The materials and reagents for the tyrosine kinase assay were from Boehringer Mannheim (Mannheim, Germany). All other reagents were of the best grade commercially available. Immunocomplexes were made visible after western blotting with the non-radioactive enhanced chemiluminescence system (ECL) and the Hyperfilm-ECL from Amersham Buchler (Braunschweig, Germany).

The cDNA for the wild-type receptor (HIR-wt) and the PKC isoform β2 were gifts from Dr. A. Ullrich (Max-Planck-Institute, Martinsried, Germany). The cDNA for the PKC isoform θ was kindly provided by Dr H. Mischak (National Institutes of Health, Bethesda, Md, USA). The cDNA for IRS-1 and the polyclonal rabbit antibody against IRS-1 were gifts from Dr M.F. White (Joslin Diabetes Center, Boston, Mass., USA). Polyclonal isoform specific PKC antibodies were purchased from Gibco. The monoclonal mouse antibody against phosphorylated tyrosine residues (PY20) were from Leinco Technologies (Ballwin, Mo., USA). The cDNA of the insulin receptor was from human origin, cDNA from IRS was from rat, those of the PKC isoforms were from mouse (θ) and bovine (β2) origin.

Preparation of serine to alanine point mutants of the insulin receptor and the kinase-negative mutant of PKCβ2. The point mutations were introduced by a method described previously [24] in the CMV promoter-based vector pRK-5 [25] containing the cDNA sequence for the wild-type human insulin receptor or PKCβ2, respectively. All amino acids were numbered as described previously [26]. The mutagenic oligos (for HIR-994: 5′-CAC CAT GCC GAA GGC GCC CTG CCC CAG-3′, for HIR-1023/25: 5′-G CTC TCG GAG ACC GGC TGC CTC GTT GAC CGT C-3′, for PKCβ2-KA: 5′-TCC TTT

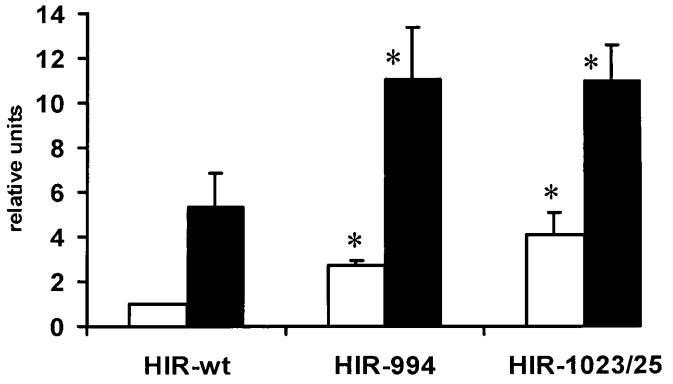

Fig. 1. Activation of insulin receptor kinase in HEK 293 fibroblasts. Cells overexpressing the wild-type insulin receptor or mutant insulin receptors were stimulated with insulin (□: basal, ■: 100 nmol/l) as described in the Methods section. Whole-cell lysates were then added to microwells coated with αIR, and after insulin receptor immobilisation kinase reactions with recombinant IRS-1 were carried out. Kinase activities are normalised for insulin binding activities measured in the same wells. Shown are means of four different cell preparations analysed in duplicate in one experiment as relative units over HIR-wt (basal). Basal and insulin stimulated kinase activity of the receptor mutants were statistically analysed separately and asterisks indicate a significantly increased activity ($p < 0.05$, $n = 4$) compared with HIR-wt in basal or stimulated state, respectively

TTC AGG ATC GCC ACA GCA TAG AGC T-3′) were purchased from Boehringer Mannheim. Introduced point mutations were verified by DNA-sequencing using the Sequenase version 2.0 kit (United States Biochemical, Cleveland, Ohio, USA) and the plasmid DNA was prepared using a Qiagen Plasmid Kit (Quiagen, Hilden, Germany).

Transient expression of HIR-wt, HIR-mutants and substrates. The HEK 293 cells (ATCC CRL 1573) were grown in Dulbecco's modified Eagle's medium (DMEM)/Nutrient Mix F12 medium (Gibco, Eggenstein, Germany) supplemented with 10% (v/v) FCS. A total of 4 μg plasmid DNA was transfected in HEK 293 cells according to a previously reported protocol [27]. Briefly, cells were grown in 6-well dishes at a density of 3×10^5 cells per well in 2 ml of medium. We mixed 4 μl supercoiled plasmid DNA (1 μg/μl) with 96 μl of 0.25 mol/l $CaCl_2$. An equal amount of 2x transfection buffer (50 mmol/l *N*,*N*-bis [2-hydroxyethyl]-2-aminoethan acid (BES), pH 6.95, 280 mmol/l NaCl, 1.5 mmol/l Na_2HPO_4) was added and after incubation for 10 min at room temperature the mixture was given dropwise to the cells. After incubation for 20 h at 37 °C and 3% CO_2, the cells were serum starved for 20 h in DMEM (1000 mg/l glucose) containing 0.5% FCS and 2 mmol/l glutamine.

Insulin receptor kinase and insulin binding activities. After stimulation with insulin, whole-cell lysates of HEK 293 fibroblasts were added to microwells coated with anti-insulin receptor (αIR) antibody [28]. After the receptor had bound to the antibody, wells were washed and kinase activity was measured in the presence of 0.3 μmol/l [γ-^{32}P]-ATP (3.7–7.4 TBq/mmol) and 2.4 μg/ml recombinant insulin receptor substrate-1 (IRS-1) (Upstate Biotechnology, New York, N.Y., USA) as described previously [29]. Wells were then washed again and insulin binding activity (defined as the amount of insulin specifically bound at 8.7 nmol/l) was analysed as described previously [28] to be sure of similar amounts of insulin receptor. Kinase activity measured as phosphate incorporation into IRS-1 was normalised for insulin binding activity. The results were expressed as relative units of the basal HIR-wt value.

Cell stimulation and lysis. Serum-starved cells were preincubated with or without 100 nmol/l of phorbol ester, 12-*O*-tetradecanoylphorbol-13-acetate, (TPA) for 30 min, stimulated with or without 100 nmol/l insulin for 5 min at 37 °C and lysed in 0.2 ml ice-cold lysis buffer (50 mmol/l HEPES pH 7.2, 150 mmol/l NaCl, 1.5 mmol/l $MgCl_2$, 1 mmol/l EGTA, 10% (v/v) glycerol, 1% (v/v) Triton X-100, 100 mmol/l NaF, 10 mmol/l sodium pyrophosphate, 100 μmol/l sodium orthovanadate, 1 mmol/l PMSF, 10 μg/ml aprotinin). Cleared cell lysates (20 min/13000 g) were analysed on a 7.5% SDS-PAGE (30 μl per lane).

Western blotting. Separated proteins were transferred to nitrocellulose membranes by semi-dry electroblotting (transfer buffer: 48 mmol/l TRIS/HCl pH 7.5; 0,04% (w/v) SDS; 39 mmol/l glycine; 20% (v/v) methanol). After transfer, nonspecific protein binding was blocked with NET-G buffer (150 mmol/l NaCl, 5 mmol/l EDTA, 50 mmol/l TRIS, 0.05% (v/v) Triton X-100 and 0.25% (w/v) gelatine, pH 7.4) for 1 h. Subsequently filters were incubated with the first antibody (αPY, αIRct, αIRS-1, αPKCβ2/θ) overnight at 4 °C. The membranes were washed four times with NET-G buffer before incubation with horseradish peroxidase conjugated anti-mouse IgG or anti-rabbit IgG for 1 h at room temperature. Immunocomplexes were made visible by ECL.

Statistical analysis Statistical analyses were done with Student's *t* test and significance is indicated by one ($p < 0.05$) or two asterisks ($p < 0.01$) in the figures.

Results

As reported earlier [21] serine to alanine exchanges at positions 994 and 1023/25 caused an increased autophosphorylation of the receptor β-subunit. Based on these findings we had speculated that serine 994 and serine 1023/25 might be part of a receptor domain which participates in inhibitory functions. Alternatively these mutations could alter the intrinsic tyrosine kinase activity of the receptor due to a conformational change. To assess the latter possibility we used an in vitro phosphorylation assay of mutant insulin receptors of recombinant IRS-1. The tyrosine kinase activity of HIR wild type increases after insulin stimulation by 5.4-fold over basal conditions (Fig. 1). A significantly higher tyrosine kinase activity of the mutants compared with HIR-wt was detected in the basal state as well as after insulin stimulation ($p < 0.05$, $n = 4$). Because we could not detect any alterations of affinity between the different receptors and IRS-1 in several coimmunoprecipitation experiments (data not shown) we do not think that the increased kinase activity is due to a changed substrate affinity.

To further investigate the signalling capacity of the receptor mutants we analysed their kinase activity to-

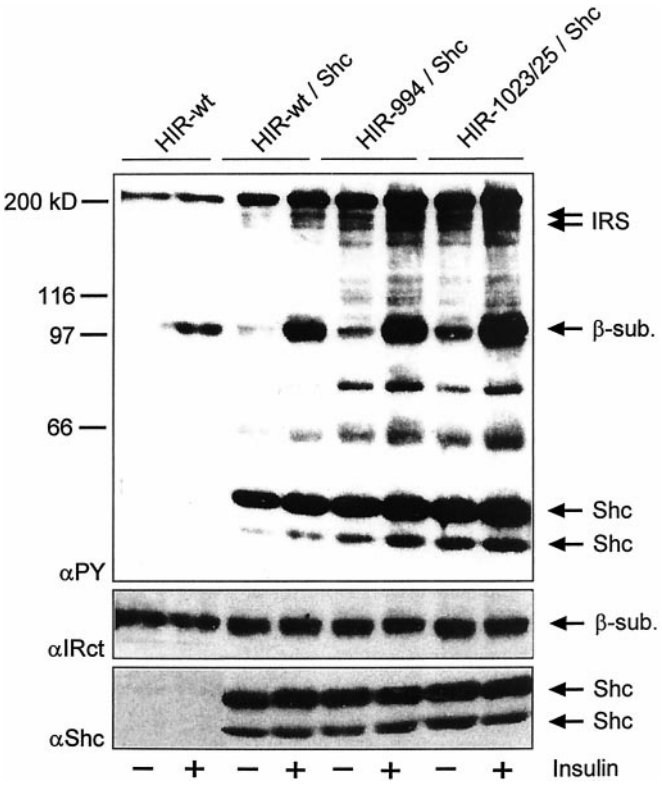

Fig. 2. Co-transfection of HIR-994 with Shc. HEK 293 cells were transiently transfected with the indicated constructs as described in the Methods section. After stimulation with 100 nmol/l insulin at 37 °C (5 min) proteins were separated by SDS-PAGE and western blots were probed with antibodies against phosphotyrosine residues (αPY, upper panel), insulin receptor β-subunit (αIRct, middle panel) or, on a separate gel loaded with equal aliquots of the lysates, against Shc (αShc, lower panel). A representative immunoblot is shown

wards the adapter protein Shc in intact cells. We co-transfected HEK 293 cells with expression constructs for HIR-wt, HIR-994 or HIR-1023/25 together with the adapter protein Shc. In lanes 1 and 2 of Figure 2 lysates of cells overexpressing the wild-type insulin receptor alone was analysed and the insulin stimulated autophosphorylation of the receptor β-subunit is visible (upper panel). When the adapter Shc was co-overexpressed (lanes 3,4) a tyrosine phosphorylation of this protein occurred which increases after insulin stimulation. With the mutant insulin receptors (lanes 5–8) not only a higher autophosphorylation of the β-subunit but a higher tyrosine phosphorylation of both Shc isoforms even at basal conditions, was seen compared with its phosphorylation level in co-transfections of HIR wild type. In addition, an increase of phosphotyrosine in the region where the IRS proteins occured was detectable in the receptor mutant lanes. It was however, not, possible to determine if these bands were IRS-1 or 2 by reblotting due to their low endogenous levels. Furthermore, two unidentified proteins of about 66000 M_r and 80000 M_r are stronger phosphorylated. To show equal amounts of expressed insulin receptor the membrane was reblotted with antibody directed against the β-subunit (Fig. 2, middle panel). For detection of Shc expression aliquots of the lysates were loaded on a separate gel and directly probed with antibodies directed against Shc (Fig. 2, lower panel).

To find whether these serine mutations are associated with an altered susceptibility to inhibitory effects of serine kinases we expressed different HIR mutants together with IRS-1 and PKCβ2 or PKCθ. This tests whether one of the insulin receptor mutants confers resistance to the inhibitory effect of phorbol ester stimulated PKCβ2 and PKCθ. Figure 3 shows representative analysis of experiments where we expressed HIR, IRS-1 and PKCβ2. A control experiment without coexpression of PKCβ2 or with a kinase-negative PKCβ2-KA showed the inhibitory effect of PKCβ2 (Fig. 3A). We termed the kinase-negative mutant as PKCβ2-KA, indicating the exchange of the lysine in the ATP-binding site of PKCβ2 to alanine (KA). Immunoblots against the insulin receptor and PKCβ2 were done to compare the amount of transfected proteins (Fig. 3A, lower two panels). Phosphotyrosine blotting made the insulin stimulated autophosphorylation and the effect of PKC activation by TPA visible (Fig. 3A, upper panel). Preincubation with the phorbol ester TPA caused an inhibition of the HIR autophosphorylation only when active PKCβ2 was coexpressed (lane 6). No inhibition was detected in the absence of PKCβ2 (lane 3) or by coexpression of the kinase-negative form PKCβ2-KA (lane 9). Next, we tested the susceptibility of the different receptor constructs to undergo the PKC-induced inhibition by co-overexpressing IRS-1, PKCβ2 and the indicated HIR mutants. Every third lane of the phosphotyrosine blot showed that stimulation with insulin and TPA reduces the HIR autophosphorylation (Fig. 3B, upper panel). This inhibition was reduced when HIR-994 and HIR-1023/25 were expressed.

A statistical analysis after densitometric scanning of six experiments is shown in Figure 4A. We corrected the differences of the expression levels by correlating the phosphotyrosine data with the receptor expression. Expression of PKCβ2 and phorbol ester stimulation reduced the insulin stimulated HIR-wt autophosphorylation to 58 %. Almost no inhibition ($p < 0.05$, $n = 6$) was observed for HIR-994 (99 % of insulin stimulated state) and HIR-1023/25 (91 %). Although other mutants including HIR-962 and HIR-1275 showed a reduced inhibition by PKC none of these serine to alanine mutants behaved statistically significantly different from HIR-wt. Figure 4B shows the quantification for the similar experiments with PKCθ. Activated PKCθ inhibited autophosphorylation of the HIR-wt to 55 % of the value obtained without activation. Again, only HIR-944 (85 % of insulin stimulated state) and HIR-1023/25 (85 %)

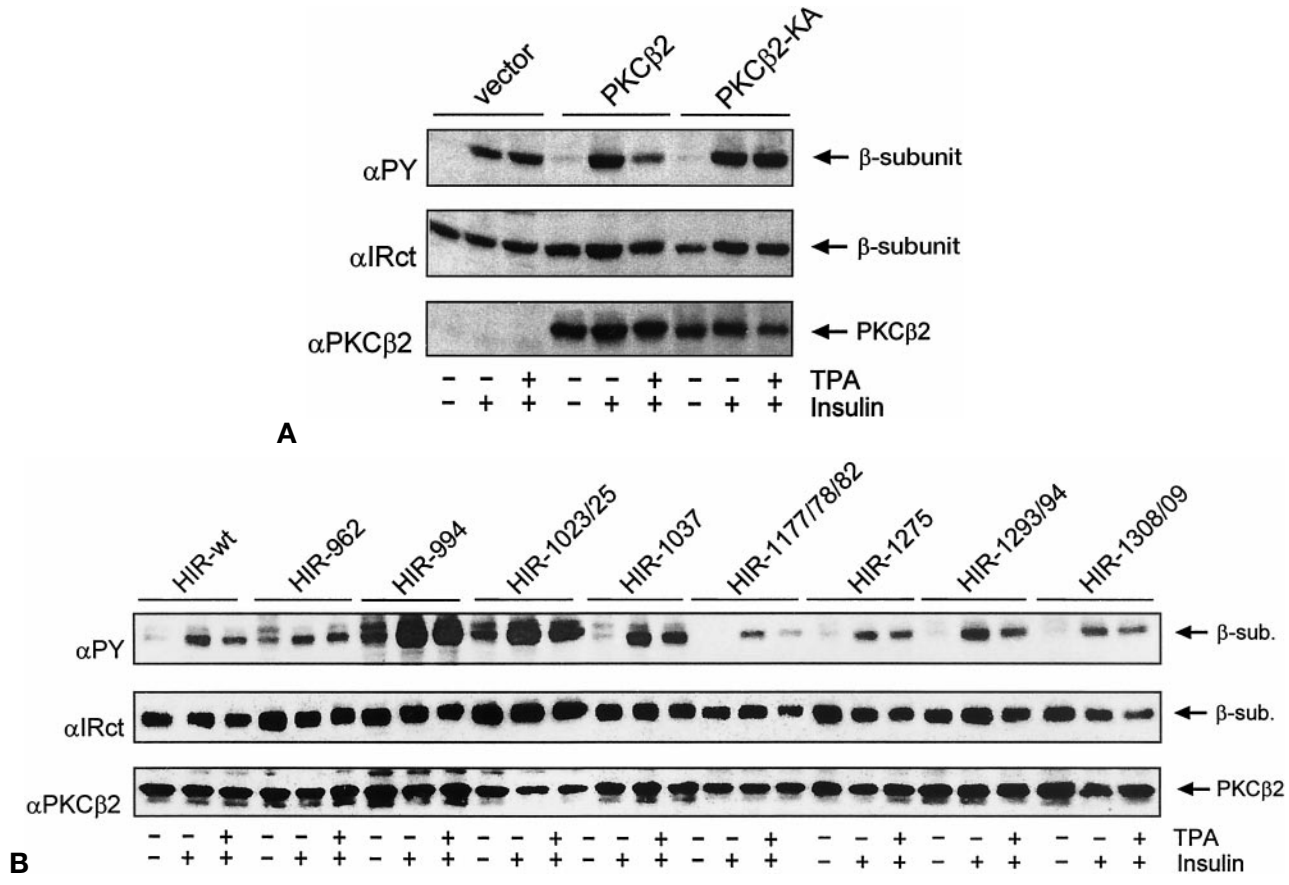

Fig. 3 A, B. Co-transfection of insulin receptors with IRS-1 and PKCβ2. HEK 293 cells transiently overexpressing (**A**) IRS-1, HIR wild type and the indicated PKCβ2-constructs or with (**B**) IRS-1, PKCβ2 and the indicated HIR-constructs, were serum starved for 20 h, preincubated with or without 100 nmol/l of TPA (30 min) and stimulated with 100 nmol/l insulin (5 min) at 37 °C. Proteins were separated by SDS-PAGE, transferred to nitrocellulose and blots were probed with antibodies against phosphotyrosine (αPY, upper panel). Similar amounts of transfected proteins were monitored with antibodies against the insulin receptor β-subunit (αIRct, middle panel) and against PKCβ2 (αPKCβ2, lower panel). An immunoblot representative of 3 (**A**) or 6 (**B**) experiments is shown

showed a significantly reduced inhibition of autophosphorylation ($p < 0.01$, $n = 5$). We conclude that the domain around the serine residues 994 and 1023/25 is important for the inhibitory effect of PKC isoforms β2 and θ.

Discussion

Based on our finding that the HIR mutants 994 and 1023/25 showed an increased tyrosine autophosphorylation when expressed in HEK 293 cells [21] we have investigated in this study the effect of these mutations on receptor tyrosine kinase activity and their susceptibility to inhibitory mechanisms. We could show a higher kinase activity towards IRS-1 and the adapter molecule Shc, which is another substrate of the insulin receptor [30]. Furthermore, a statistically significant reduced inhibition of the mutant receptors by protein kinase C isoforms β2 and θ was observed.

Among the inhibitory proteins for insulin signalling tyrosine phosphatases and serine kinases are of predominant interest. Several protein tyrosine phosphatases have been shown to function as negative modulators of insulin signalling [31–35]. We concentrated on the negative feedback loop through serine kinases, i. e. protein kinase C. Our co-transfection experiments with the protein kinase C β2 and θ suggest that the serines at position 994 and 1023/25 are involved in the interaction of the receptor with these PKC isoforms. The mechanism of this interaction is not clear. When we previously studied the interaction of HIR-wt and different PKC isoforms in the absence of IRS-1 we observed that only the PKC isoforms β1 and β2 exert an inhibitory effect on the insulin receptor [22]. When IRS-1 was co-transfected with the HIR and PKC isoforms, not only a stronger inhibitory effect of PKCβ was observed but also other PKC isoforms became able to inhibit the insulin receptor [23]. These studies suggested that IRS-1 is necessary for the inhibitory effect of some PKC isoforms. It is not clear whether IRS-1 is required as a co-factor or

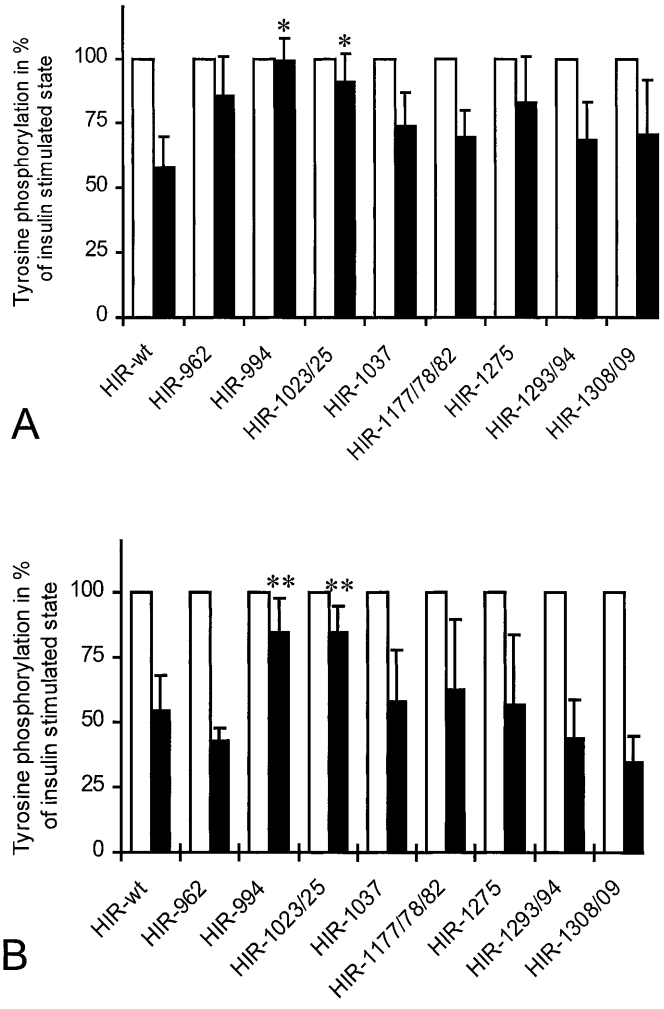

Fig. 4 A, B. Densitometric analysis of the co-transfection experiments. After scanning densitometry of each experiment where the different insulin receptor constructs were co-transfected with IRS-1 and (**A**) PKCβ2 or (**B**) PKCθ the insulin stimulated receptor tyrosine phosphorylation was set as 100 % for each receptor construct (white bars). Black bars show the reduced tyrosine phosphorylation for each different receptor construct after preincubation with 100 nmol/l TPA (mean in % ± SEM). Asterisks indicate significant alterations in (**A**) ($p < 0.05$, $n = 6$) and in (**B**) ($p < 0.01$, $n = 5$)

whether IRS-1 itself mediates the inhibitory effect. There is, however, increasing evidence that serine phosphorylation of IRS-1 caused by different serine kinases including MAPK [36], glycogen synthase kinase 3 (GSK3) [37], PI3 [38] and PKC isoforms [39] is required. Recently, we were able to show that PKCθ phosphorylates IRS-1 on serine residues [23].

It is not known whether HIR-994 is a phosphorylation site for PKC although a phosphorylation of serines 1023/25 by PKC has already been shown [16]. Our observation that even the receptor mutated at serine position 962 was less well inhibited by PKCβ2 leads us to speculate that the domain around these residues is important for PKC mediated receptor inhibition. In conclusion, our data suggest that the inhibitory activity of PKC isoforms β2 and θ is disturbed when serines 994 or 1023/25 of the insulin receptor are mutated.

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