Identification of serines-967/968 in the juxtamembrane region of the insulin receptor as insulin-stimulated phosphorylation sites

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A line of Chinese hamster ovary cells overexpressing protein kinase C α was transfected with cDNAs encoding either the wild-type human insulin receptor or one of two mutant insulin receptors with either Ser-967 and -968 or -974 and -976 in the juxtamembrane region changed to alanine. Both mutant receptors exhibited normal insulin-activated tyrosine kinase activity as assessed by either autophosphorylation or insulin-stimulated increases in anti-phosphotyrosine-precipitable phosphatidylinositol 3-kinase. The wild-type and mutant insulin receptors were also examined for serine and threonine phosphorylation in response to insulin and activation of protein kinase C. To visualize Ser/Thr-phosphorylation sites of the receptor better in response to insulin, the receptor from *in vivo*-labelled insulin-treated cells was first treated with a tyrosine-specific phosphatase to remove all tyrosine phosphorylation. Phosphopeptides

from the three receptors were analysed by high-percentage polyacrylamide/urea gel electrophoresis and two-dimensional t.l.c. The mutant receptor lacking Ser-967 and -968 but not the mutant lacking Ser-974 and -976 was found to be missing phosphorylated peptides in response to insulin and, to a lesser extent, after activation of protein kinase C. However, the insulin-stimulated increase in anti-phosphotyrosine-precipitable phosphatidylinositol 3-kinase was inhibited to the same extent by activation of protein kinase C in cells expressing the two mutant receptors as in cells expressing the wild-type receptor. These results indicate that these four serine residues in the juxtamembrane region are not major regulatory sites of the intrinsic tyrosine kinase activity of the insulin receptor by protein kinase C, although Ser-967 and/or -968 appear to be phosphorylated in response to insulin.

INTRODUCTION

Insulin induces extensive tyrosine phosphorylation of the cytoplasmic domain of the β -subunit of its receptor. At least six tyrosines appear to be phosphorylated, including one in the juxtamembrane region (Tyr-972), three in the kinase domain (Tyr-1158, -1162 and -1163) and two in the carboxy tail of the receptor (Tyr-1328 and -1334) (Tavare and Denton, 1988; Avruch et al., 1990; Feener et al., 1993). The tyrosine phosphorylation of residues in the kinase domain appears to greatly activate the ability of the receptor to phosphorylate other proteins, both *in vitro* and *in vivo*, and to make the receptor kinase independent of the presence of insulin (Ellis et al., 1986; Avruch et al., 1990; Wilden et al., 1992). The tyrosine phosphorylation of residues in the carboxy tail of the receptor appears to potentiate the ability of the receptor to stimulate some responses but not others in certain cell types (Ando et al., 1992; Dickens et al., 1992; Takata et al., 1992). Phosphorylation of the juxtamembrane tyrosine appears not to affect receptor autophosphorylation but to affect the ability of the receptor to mediate subsequent biological responses and to phosphorylate various endogenous substrates including insulin receptor substrate 1 (IRS-1) (White et al., 1988; Feener et al., 1993).

In addition to this tyrosine phosphorylation, the insulin receptor β -subunit is also extensively phosphorylated on serine and threonine residues (Avruch et al., 1990; Pillay et al., 1991). The Ser/Thr phosphorylation of the receptor also occurs in response to insulin treatment of cells as well as when cells are treated with activators of either protein kinase C (PKC) or cyclic AMP kinase (Jacobs and Cuatrecasas, 1986; Stadtmauer and Rosen, 1986; Takayama et al., 1988). At least one threonine

(Thr-1348) and two serines (Ser-1305/1306) in the carboxy tail of the receptor have been found to be phosphorylated *in vitro* by purified PKC and *in vivo* after treatment of cells with either insulin or activators of PKC (Koshio et al., 1989; Lewis et al., 1990a,b; Tavare et al., 1991; Ahn et al., 1993). The insulin receptor has also been shown to be phosphorylated *in vitro* by cyclic AMP kinase (Roth and Beaudoin, 1987), a casein kinase I-like enzyme (Rapuano and Rosen, 1991) and an insulin-stimulated Ser/Thr kinase that associates with the receptor but has yet to be purified (Czech et al., 1988; Smith et al., 1988). It has also been reported that the insulin receptor itself may be capable of Ser/Thr autophosphorylation (Baltensperger et al., 1992). In contrast with the well-defined effects of tyrosine phosphorylation of the receptor on receptor functions, the effects of Ser/Thr phosphorylation have been more controversial. Although some initial reports suggested that Ser/Thr phosphorylation might affect insulin binding, most subsequent reports have not confirmed this (Grunberger, 1991; Haring, 1991). Ser/Thr phosphorylation of the receptor has also been reported in some studies to affect the receptor's intrinsic tyrosine kinase activity when assayed *in vitro* with artificial substrates (Takayama et al., 1988; Karasik et al., 1990). In recent studies this could not be confirmed, although it was found that the insulin-stimulated increase in anti-phosphotyrosine-precipitable phosphatidylinositol 3-kinase activity was decreased when the receptor was extensively Ser/Thr phosphorylated by activation of PKC (Chin et al., 1992; Coghlan and Siddle, 1993). The inhibitory effect of Ser/Thr phosphorylation of the insulin receptor is of potential importance in the desensitization of the receptor, either in response to insulin itself or through the activation of other signalling systems by counter regulatory hormones (Treadway

Abbreviations used: IRS-1, insulin receptor substrate-1; CHO, Chinese hamster ovary; PMA, phorbol 12-myristate 13-acetate; PKC, protein kinase C; PTP 1b, protein tyrosine phosphatase 1b; GST, glutathione transferase.

The amino acid numbering corresponds to that of Ebina et al. (1985).

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et al., 1989; Issad et al., 1992). In addition, excessive Ser/Thr phosphorylation of the receptor, possibly as a result of activation of PKC, could even play a role in the decreased receptor kinase activity observed in many non-insulin-dependent diabetic patients with insulin resistance (Caro et al., 1989; Thies et al., 1990; Haring, 1991; Inoguchi et al., 1992).

A further delineation of the kinases responsible for this Ser/Thr phosphorylation of the receptor, as well as the determination of the particular amino acid residues involved, could therefore be important in unravelling the pathogenesis of non-insulin-dependent diabetes. In the present study we report on the results of the mutagenesis of all four serine residues in the juxtamembrane region of the insulin receptor. These serines were chosen because, as noted above, this region appears to play a critical role in the recognition of endogenous substrates such as IRS-1. In addition, recent studies of Feener et al. (1993) indicated that serine phosphorylation occurred in this region of the receptor. The present studies indicate that Ser-967 and/or -968 are phosphorylated in response to insulin and, to a lesser extent, on activation of PKC. However, phosphorylation of these serines did not mediate the decrease in insulin-stimulated anti-phosphotyrosine-precipitable phosphatidylinositol 3-kinase observed after activation of PKC (Chin et al., 1992).

MATERIALS AND METHODS

Site-directed mutagenesis and construction of expression vectors

The wild-type human insulin receptor cDNA (Ellis et al., 1986) was subcloned into pBluescript II SK plasmid (Stratagene) and used as the template to generate mutants S967/968A and S974A. Oligonucleotide-directed mutagenesis was carried out as described by Kunkel et al. (1987). Chemically synthesized oligonucleotides used for mutagenesis are as follows (the mismatched bases are underlined): S967/968A, 5'-ACCGCTTTACGCTGCAGCAAACCCTGAG-3' (new *Pst*I site); S974A, 5'-CCTGAGTATCTCGGTGCCAGTGA-3' (new *Ban*I site). To generate mutant S974/976A, a 2.4 kb *Bam*HI-*Xba*I fragment was isolated from the S974A mutant cDNA and subcloned into pBluescript II SK plasmid. Double-stranded DNA site-directed mutagenesis was carried out by using the Transformer Site-Directed Mutagenesis Kit (Clontech). The oligonucleotide used for mutagenesis was as follows (the mismatched bases are underlined): 5'-CTGAGTATCTCGCTGCCGCTGATGTGTTTC-3'. The trans oligonucleotide used was: 5'-GGTCGACGGTCCGCGATAAAGCTTGA-3' (to remove the *Cla*I site in the multiple cloning site of the pBluescript plasmid). The recombinant plasmids containing the mutant receptor cDNAs were used to transform DH5 α and the mutations and proper construction of the vectors were confirmed by DNA sequencing the mutated regions. The mutant cDNAs were subcloned into the SR α expression vector (Takebe et al., 1988), and the recombinant plasmids were used for transfections.

Expression of PKC α in Chinese hamster ovary (CHO) cell lines

Transfections were carried out as described previously (Chin et al., 1992). In brief, 5×10^6 CHO cells were grown in 100-mm-diam. plates and incubated overnight at 35 °C and 3% CO $_2$. pECE-PKC α (a gift from Dr. P. Parker, ICRF Laboratory, London) (20 μ g) and 1 μ g of pSV2 $_{neo}$ were allowed to form a calcium co-precipitate. The precipitate was added dropwise to the cell medium and the cells were incubated for 4 h at 35 °C and 3% CO $_2$. After a glycerol shock, the cells were incubated overnight at 37 °C and 5% CO $_2$. The cells were then split 1:10

and placed in Ham's F-12 medium containing 10% (v/v) newborn-calf serum (Gibco), 100 μ g/ml streptomycin and 400 μ g/ml G418 (Gibco). Positive colonies were identified by [3 H]phorbol 12,13-dibutyrate binding and PKC activity as described (Chin et al., 1992) and then cloned by limiting dilution.

Expression of the wild-type and mutant insulin receptors in CHO/PKC cell lines

Transfections were carried out as described above. SR α insulin receptor (20 μ g) and 1 μ g of PBS $_{pac\Delta p}$ (de la Luna et al., 1988) were used in the transfections. Transfectants were selected with 8 μ g/ml puromycin. Positive colonies were identified by Western blotting with anti-phosphotyrosine antibody RC-20 (Signal Transduction Laboratories) after insulin stimulation and confirmed by Western blotting with anti-(insulin receptor) antibody.

Expression and purification of protein tyrosine phosphatase 1b (PTP 1b)

Plasmid pGEXKG, which encodes rat brain PTP 1b fused to glutathione transferase (GST), was a gift from Dr. A. Saltiel (Warner-Lambert Co., Ann Arbor, MI, U.S.A.). For expression and purification of the GST-PTP 1b fusion protein, the plasmid was used to transform *Escherichia coli* strain DH5 α . Overnight cell cultures (100 ml) containing the plasmid were diluted 1:10 and grown at 28 °C for 1 h and 20 min. Expression of GST-PTP 1b fusion protein was induced by addition of isopropyl β -D-thiogalactoside to a final concentration of 1 mM. After a further 3.5 h, the cell culture was harvested, washed with 10 mM Tris/HCl, pH 8.0, and the cell pellet was resuspended in lysis buffer containing 50 mM Tris/HCl, pH 7.5, 1 mM dithiothreitol, 5 mM EDTA, 50 mM KCl, 1 mM phenylmethanesulphonyl fluoride, 1% (v/v) Triton X-100 and 1 mg/ml lysozyme. After 30 min at 0 °C, the lysate was sonicated for 5 min at 0 °C. The cell debris was removed by centrifugation at 12000 *g* for 15 min. The GST-PTP 1b fusion protein was purified by incubating the supernatant with 1 ml of glutathione-agarose (Pharmacia) overnight at 4 °C. After the agarose beads had been washed three times with PBS (5 mM Na $_2$ HPO $_4$, pH 7.4, 15 mM NaCl), the GST-PTP 1b fusion protein was eluted with 50 mM Tris/HCl, pH 8.0, containing 10 mM GSH. The purified enzyme was stored at -20 °C in 20% (v/v) glycerol.

Radiolabelling and PTP 1b treatment of 32 P-labelled insulin receptor *in vivo*

Monolayers of cells were grown in 100-mm-diam. plates to confluence. After incubation in phosphate-free Krebs-Ringer bicarbonate buffer (KRBB; 20 mM Hepes, pH 7.6, 3 mM CaCl $_2$, 5 mM KCl, 7 mM NaHCO $_3$, 107 mM NaCl, 1 mM MgSO $_4$, 10 mM glucose, 0.1% BSA) for 30 min at 37 °C, the cells were radiolabelled with 0.3 mCi of carrier-free [32 P]orthophosphate/plate for 3 h at 37 °C. Insulin (10 nM) was added during the last 8 min of radiolabelling. The cells were then placed on ice, washed twice with ice-cold KRBB, and lysed with 0.6 ml/plate lysis buffer containing 50 mM Hepes, pH 7.6, 150 mM NaCl, 1% Triton X-100, 10 mM NaF, 50 mM β -glycerophosphate, 1 mg/ml bacitracin, 1 mM phenylmethanesulphonyl fluoride and 1 mM *N*-ethylmaleimide. Cell debris was pelleted at 14000 *g* for 10 min and the supernatant fraction was immunoprecipitated overnight at 4 °C with 5 μ g each of the anti-(insulin receptor) monoclonal antibodies 5D9 and 29B4 bound to Protein G-agarose. The immunoprecipitates were washed twice with buffer A (20 mM Na $_2$ HPO $_4$, pH 8.6, 0.5% Triton X-100, 0.1% SDS, 0.02%

Figure 1 Expression of the wild-type and mutant insulin receptors

(a) Immunoblotting of the insulin receptors. Lysates of insulin-treated parental CHO cells (lane A), cells overexpressing the wild-type receptor (lane B) or cells overexpressing mutant S967/9768A (lane C) or mutant S974/976A (lane D) were immunoprecipitated with monoclonal anti-receptor antibodies and the precipitates were electrophoresed on 10% (w/v) polyacrylamide/SDS gels, transferred to nitrocellulose and immunoblotted with a monoclonal antibody to the β -subunit of the insulin receptor. (b) Anti-phosphotyrosine immunoblotting. The same blot as in (a) except that it was probed with anti-phosphotyrosine antibody RC-20.

Figure 2 Treatment of receptor with PTP 1b

(a) Analysis of *in vivo*-labelled receptor. Metabolically labelled cells overexpressing the wild-type insulin receptor were treated with 10 nM insulin for 8 min as indicated. The insulin receptor from the cells was immunoprecipitated and treated with PTP 1b as indicated. The precipitates were electrophoresed on a 10% (w/v) polyacrylamide/SDS gel and transferred to Immobilon P; its autoradiogram is shown. (b) Analyses of PTP 1b-treated receptor by immunoblotting. The same blot as shown in (a) was immunoblotted with the anti-phosphotyrosine antibody RC-20. (c) Phosphoamino acid analysis of the PTP 1b-treated receptor. *In vivo*-labelled receptor from the insulin-treated cells was treated with or without PTP 1b, hydrolysed and the phosphoamino acids were determined.

Na_3PO_4 containing 1 M NaCl and 0.1% BSA and then twice with buffer A containing 0.15 M NaCl.

For PTP 1b treatment, the immunoprecipitates were resuspended in a buffer containing 25 mM imidazole/HCl, pH 7.0, 10 mM dithiothreitol, 0.15 M NaCl, 1 mg/ml BSA and 0.5 μM okadaic acid (Calbiochem). The reaction mixture was incubated at 30 $^\circ\text{C}$ for 15 min. Optimal conditions for removal of all the phosphotyrosine were previously determined by varying the amounts of enzyme and the time of incubation.

Figure 3 Analysis of the phosphorylated tryptic peptides of the wild-type and mutant insulin receptors by high-percentage polyacrylamide/urea gel electrophoresis

In vivo-labelled cells were treated with either 10 nM insulin (a, lanes B–D) or 1.6 μM PMA (a, lane A; b, lanes A–C). The insulin receptors from cells overexpressing the wild-type receptor (a, lanes A and B; b, lane A), mutant S967/968A (a, lane C; b, lane B) or mutant S974/976A (a, lane D; b, lane C) were immunoprecipitated, treated with the PTP 1b and cleaved with trypsin as described in the Materials and methods section. The resulting tryptic peptides were analysed by high-percentage polyacrylamide/urea gel electrophoresis.

SDS/PAGE and Western-Blot analysis

SDS/PAGE was performed by the procedure of Laemmli (1970) using 10% (w/v) polyacrylamide. After electrophoresis, proteins were electrophoretically transferred to nitrocellulose membranes for Western blotting or Immobilon P for phosphoamino acid analysis. The immunoblots were blocked for 1 h at room temperature in a solution containing 10 mM Tris/HCl, pH 7.5, 154 mM NaCl, 3% BSA and 0.1% Tween 20 and then incubated overnight at 4 $^\circ\text{C}$ with monoclonal anti-receptor antibody 2H2 directed against the β -subunit of the insulin receptor (a gift from Dr. K. Shii, Hyogo Institute, Aitashi, Japan). After incubation with horseradish peroxidase-conjugated anti-(mouse IgG) antibody for 1 h at room temperature, the membrane was developed with the ECL kit (Amersham).

Phosphoamino acid analysis

The insulin receptor on the Immobilon P membrane was hydrolysed under vacuum for 2.5 h at 110 $^\circ\text{C}$ in 1 ml of 6 M HCl (Pierce Chemical Co.). The membrane was rehydrated by the addition of 1 drop of methanol, and the amino acids were eluted by incubating the membrane with 1 ml of water for 1 h at room temperature or overnight at 4 $^\circ\text{C}$. After lyophilization, the sample was resuspended in 5 μl of phosphoamino acid standards containing phosphotyrosine, phosphoserine and phosphothreonine and spotted on to t.l.c. plates. Thin-layer electrophoresis was carried out at 1500 V for 55 min in acetic acid/pyridine/water (10:1:189, by vol.). The plates were dried and exposed to X-ray films.

Tryptic digestion, high-percentage PAGE and two-dimensional phosphopeptide mapping

Tryptic digestion of ^{32}P -labelled human insulin receptor was conducted as described by Luo et al. (1991). In brief, the

Figure 4 Two-dimensional mapping of the phosphorylated tryptic peptides of the wild-type (a) and mutant S967/968A (b) insulin receptors after insulin treatment

Immunoprecipitated receptor from metabolically labelled cells was treated with PTP 1b, cleaved with trypsin and analysed as described in the Materials and methods section. A schematic diagram of the observed peptides is shown in (c). Peptide T is so called as it migrates in a position that corresponds to that of a peptide containing phosphothreonine although this peptide has not been shown to contain phosphothreonine in the present work.

radiophosphorylated insulin receptor was subjected to SDS/PAGE, blotted on to nitrocellulose membranes and digested with 20 μ g of TosPheCH₂Cl ('TPCK')-treated trypsin (Worthington) in a freshly prepared 50 mM NH₄HCO₃ buffer at 37 °C for 18 h. An additional 10 μ g of TosPheCH₂Cl-treated trypsin was added and the reaction mixture was incubated for a further 6 h. The tryptic digests were lyophilized, resuspended in a gel loading buffer containing 0.125 M Tris/HCl, pH 6.8, 6 M urea and Bromophenol Blue. High-percentage alkaline polyacrylamide gels were prepared as described by West et al. (1984). Electrophoresis was carried out overnight at a constant current of 10 mA. The gel was dried under vacuum and autoradiographed. For two-dimensional tryptic phosphopeptide mapping, the lyophilized sample was resuspended in 2–3 μ l of water containing 5 mg/ml dinitrophenyl-lysine and 1 mg/ml xylene cyanol FF and spotted on a 100 μ m 20 cm $\times$ 20 cm cellulose t.l.c. plate (E. Merck, catalogue no. 5716). First-dimension separation was carried out at pH 3.5 in acetic acid/pyridine/water (10:1:189, by vol.) (Boyle et al., 1991). The electrophoresis was conducted at 400 V for 2 h. The second-dimension t.l.c. was carried out for 8–10 h in a buffer containing butan-1-ol/pyridine/acetic acid/water (15:10:3:12, by vol.). The plates were dried and autoradiographed.

RESULTS

For the present studies, a line of CHO cells overexpressing PKC α was developed. By phorbol binding, these cells expressed approx. seven times as much PKC as the parental CHO cells. This cell line was then transfected with cDNAs encoding the wild-type human insulin receptor, a mutant insulin receptor with Ser-967 and -968 changed to alanine (S967/968A) or a mutant insulin receptor with Ser-974 and -976 changed to alanine (S974/976A). Cell lines overexpressing the three different receptors were then characterized. By Western blotting with an antibody to the β -subunit (Figure 1a), all three cell lines were found to express high levels of insulin receptor. The cells expressing the wild-type receptor and mutant S974/976A were found to contain approx. the same amount of receptor, whereas mutant S967/968A expressed approx. two to three times less receptor (Figure 1a). Both mutant receptors appeared to be processed normally, as the fraction of proreceptor and the sizes of their β -subunits were normal (Figure 1a). The receptor tyrosine kinase activities of the two mutant receptors also appeared to be unaffected, as insulin stimulated comparable increases in the extent of tyrosine autophosphorylation in the three receptors (Figure 1b).

To assess the insulin-stimulated serine phosphorylation of the

Figure 5 Two-dimensional mapping of the phosphorylated tryptic peptides of the wild-type (a) and mutant S967/968A (b) insulin receptors after activation of PKC

Immunoprecipitated receptors from metabolically labelled cells treated with $1.6 \mu\text{M}$ PMA were cleaved with trypsin and analysed as described in the Materials and methods section. A schematic diagram of the observed peptides is shown in (c).

receptors *in vivo*, a method was sought that would allow a better examination of the serine-phosphorylation sites without the complication of insulin-stimulated tyrosine phosphorylation. To this end, receptor from insulin-treated cells was immunoprecipitated and then treated with a recombinantly produced tyrosine-specific phosphatase in the presence of an inhibitor of serine phosphatases, okadaic acid (Cohen et al., 1990). This procedure was found to remove all of the detectable tyrosine phosphorylation without affecting the extent of serine phosphorylation of the receptor (Figure 2). For *in vivo*-labelled cells, approx. 60 % of the counts were removed from the receptor from insulin-treated cells and no decrease was observed in the receptor from phorbol 12-myristate 13-acetate (PMA)-stimulated cells.

The wild-type and two mutant receptors from *in vivo*-labelled insulin-treated cells were isolated by immunoprecipitation, treated with the tyrosine-specific phosphatase and digested with trypsin. The labelled peptides were analysed by high-percentage polyacrylamide/urea gel electrophoresis. Wild-type receptor from insulin-treated cells exhibited three major (peptides 1i–3i) and three minor (peptides 4i–6i) bands (Figure 3a). One major (2i) and two minor (4i and 6i) bands were missing from mutant S967/968A. Mutant S974/976A had the same phosphopeptide bands as the wild-type receptor (Figure 3a). Wild-type receptor from PMA-stimulated cells exhibited two major bands (peptides 1t and 3t) with the same mobility as two bands (peptides 1i and 3i) from the wild-type receptor of insulin-treated cells (Figure 3). Mutant receptor S974/976A from PMA-stimulated cells exhibited the same pattern as the wild-type receptor, whereas mutant S967/968A lacked one of the minor phosphopeptide bands (peptide 4t) present in the wild-type receptor (Figure 3b).

To confirm the differences in the pattern of phosphopeptide tryptic bands observed in polyacrylamide/urea gel electro-

phoresis between the wild-type and mutant S967/968A receptors, tryptic peptides from these two receptors were also analysed by two-dimensional t.l.c. By this technique, mutant S967/968A was also observed to lack one major phosphopeptide (peptide b) in insulin-treated cells (Figure 4) and two minor spots in PMA-treated cells (Figure 5).

The two mutant receptors were also tested for their ability to mediate the insulin-stimulated increase in anti-phosphotyrosine-precipitable phosphatidylinositol 3-kinase activity. The mutant receptors were found to behave like the wild-type receptor in this assay (Figure 6). Although the serines in the juxtamembrane region did not appear to be major sites of PMA-stimulated phosphorylation (Figures 3 and 5), we also verified that the mutant receptors were regulated in the same way as the wild-type receptor after activation of PKC. To this end, the three cell lines were treated with various concentrations of insulin in the presence or absence of PMA, lysed and the lysates were adsorbed with anti-phosphotyrosine antibodies. The resulting precipitates were assayed for phosphatidylinositol 3-kinase activity. PMA treatment caused an approx. 60–70 % inhibition of the insulin-stimulated increase in this activity in the cells expressing the wild-type receptor as well as the cells expressing the two mutant receptors (Figure 6).

DISCUSSION

Although extensive studies have documented the existence of at least six tyrosine autophosphorylation sites in the insulin receptor (Avruch et al., 1990), much less success has been reported on the identification of the many serine- and threonine-phosphorylation sites in the receptor. The possibility that Ser/Thr phosphorylation of the receptor regulates its intrinsic tyrosine kinase activity and

Figure 6 PMA-mediated inhibition of the insulin-stimulated increase in anti-phosphotyrosine-precipitable phosphatidylinositol 3-kinase in cells expressing (a) wild-type, (b) mutant S967/968A or (c) mutant S974/976A insulin receptors

The indicated cells were treated with the various concentrations of insulin in the presence (▲) or absence (○) of 1.6 μ M PMA. For each cell type, the results shown have been normalized to the amount of phosphatidylinositol 3-kinase activity present in the precipitates from cells treated with 10 nM insulin in the absence of PMA.

subsequent signal-transducing abilities after either heterologous and/or homologous desensitization and possibly in type-II diabetes makes the identification of these sites an important goal.

In the present work we report on our attempts to identify serine-phosphorylation sites in the juxtamembrane region of the receptor. Previous studies have identified threonine (Thr-1348) and serine (Ser-1305/1306) phosphorylation sites in the carboxy tail of the receptor (Koshio et al., 1989; Lewis et al., 1990a,b; Tavaré et al., 1991; Ahn et al., 1993). However, recent studies have indicated that the juxtamembrane region plays a critical role in determining the ability of the receptor to phosphorylate an endogenous substrate (IRS-1) and mediate biological signalling and that both tyrosine- and serine-phosphorylation sites are present in this region of the receptor (White et al., 1988; Feener et al., 1993). Thus phosphorylation of one of the four serines (Ser-967, -968, -974 and -976) in this region could affect receptor signalling.

In the present study, we report on the characterization of two mutant receptors in which the four serines have been mutated. In one mutant, Ser-967 and -968 have been changed to alanine, and in the other mutant, Ser-974 and -976 have been mutated. Both mutant receptors appear to be expressed and processed normally (Figure 1). In addition, both mutant receptors appear to auto-phosphorylate normally and to mediate the phosphorylation of an endogenous substrate as assessed by the insulin-stimulated increase in anti-phosphotyrosine-precipitable phosphatidylinositol 3-kinase activity (Figures 1 and 6). These results suggest that the structure of these two mutant receptors has not been drastically altered by mutagenesis.

The identification of insulin-stimulated serine-phosphorylation sites in the insulin receptor is complicated by the extensive tyrosine phosphorylation that accompanies insulin treatment. In the present studies we show that the tyrosine phosphorylation can be mostly eliminated without affecting the serine phosphorylation of the receptor by the use of a recombinantly produced tyrosine phosphatase (Figure 2). By utilizing this procedure, we can show that insulin treatment of cells results in the phosphorylation of multiple serine sites in the receptor (Figure 3). A major fraction (approx. 20%) of the serine phosphorylation is lost in the mutant receptor lacking Ser-967/968. This correlates with the loss of one major site by two-dimensional t.l.c. (Figure 4) and one major and two minor bands on high-percentage polyacrylamide/urea gel electrophoresis (Figure 3). The presence of three bands on this gel that are affected by the mutagenesis could be due to heterogeneity in the charge of this peptide and/or its partial cleavage, as these gels separate by a mixture of charge and size. In regard to this latter possibility, it should be noted that both the arginine preceding and the one following the juxtamembrane tryptic peptide are poor sites for trypsin cleavage, as they are either in a cluster with other basic residues or followed by a glutamic acid respectively (Boyle et al., 1991). The inability to detect the two minor peptides by two-dimensional t.l.c. could be due to these peptides being poorly resolved in this system. In summary, we have found by two different procedures that a mutant receptor lacking Ser-967 and -968 exhibits a loss in phosphopeptide(s) observed after insulin stimulation. The simplest explanation for these results is that Ser-967 and/or -968 are phosphorylated in response to insulin treatment. In contrast, mutant S974/976A did not show a loss of any of the major serine-phosphorylated bands (Figure 3), indicating that these serines are not major sites for insulin-stimulated phosphorylation.

Activation of PKC in cells has also been shown to result in an increase in the Ser/Thr phosphorylation of the receptor (Jacobs and Cuatrecasas, 1986; Takayama et al., 1988; Pillay et al., 1991). To ensure a high level of Ser/Thr phosphorylation of the receptor by this kinase, we have utilized a line of CHO cells which overexpresses PKC α to express the mutant and wild-type

insulin receptors. Previous studies have shown that cells over-expressing this enzyme exhibit 3- to 4-fold higher levels of Ser/Thr phosphorylation of the receptor in response to activators of PKC (Chin et al., 1992). Even with this high level of Ser/Thr phosphorylation, the mutant S967/968A only exhibited a loss of a minor phosphorylated peptide in PMA-treated cells (Figure 3). These results indicate that these serines are not major phosphorylation sites in cells with activated PKC. Mutants S974/976A did not exhibit any difference in phosphorylation in response to PMA (Figure 3), indicating that these two serines are not phosphorylated in response to activation of PKC.

Although the serines in the juxtamembrane region did not appear to be major phosphorylation sites in response to activation of PKC, it is still possible that they are important regulatory phosphorylation sites. Although some reports have indicated that a decrease in receptor kinase activity could be observed *in vitro* after isolation of the receptor from cells with activated PKC (Takayama et al., 1988; Karasik et al., 1990), more recent studies indicate that a better monitor of the inhibitory effect of excessive Ser/Thr phosphorylation of the receptor is a decrease in the ability of the receptor to mediate the tyrosine phosphorylation of an endogenous substrate. This can be most readily monitored by measuring the amount of phosphatidylinositol 3-kinase in anti-phosphotyrosine precipitates from insulin-treated cells (Chin et al., 1992). In agreement with these previous studies, a decrease in insulin-stimulated anti-phosphotyrosine-precipitable phosphatidylinositol 3-kinase activity was observed after PMA treatment of cells overexpressing the wild-type insulin receptor and PKC α (Figure 6). The extent of inhibition of this response was approximately the same in the cells expressing the two mutant receptors (60–70%) as was observed in the cells expressing the wild-type receptor (Figure 6). These results are consistent with the above described findings, indicating that the serines in the juxtamembrane region are not phosphorylated to a great extent after activation of PKC. Additional studies are therefore required to identify the other serine-phosphorylation sites of the receptor induced by activation of PKC and to determine which, if any, of these are important in regulating the intrinsic tyrosine kinase activity of the receptor.

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REFERENCES

- Ahn, J., Donner, D. B. and Rosen, O. M. (1993) *J. Biol. Chem.* **268**, 7571–7576
- Ando, A., Momomura, K., Tobe, K., Yamamoto-Honda, R., Sakura, H., Tamori, Y., Kaburagi, Y., Koshio, O., Akanuma, Y., Yazaki, Y., Kasuga, M. and Kadowaki, T. (1992) *J. Biol. Chem.* **267**, 12788–12796
- Avruch, J., Tornqvist, H. E., Gunsalus, J. R., Yorkow, E. J., Kyriakis, J. M. and Price, D. J. (1990) *Handbook of Experimental Pharmacology: Insulin*, pp. 313–366, Springer-Verlag, Berlin and Heidelberg
- Baltensperger, K., Lewis, R. E., Woon, C.-W., Vissavajhala, P., Ross, A. H. and Czech, M. P. (1992) *Proc. Natl. Acad. Sci. U.S.A.* **89**, 7885–7889
- Boyle, W. J., van der Geer, P. and Hunter, T. (1991) *Methods Enzymol.* **201**, 110–149
- Caro, J. F., Dohm, L. G., Pories, W. J. and Sinha, M. K. (1989) *Diabetes/Metab. Rev.* **5**, 665–689
- Chin, J. E., Dickens, M., Tavare, J. M. and Roth, R. A. (1992) *J. Biol. Chem.* **268**, 6338–6347
- Coghlan, M. P. and Siddle, K. (1993) *Biochem. Biophys. Res. Commun.* **193**, 371–377
- Cohen, P., Holmes, I. D., Roth, R. A. and Tsukitani, Y. (1990) *Trends Biochem. Sci.* **5**, 98–102
- Czech, M. P., Klarlund, J. K., Yagaloff, K. A., Bradford, A. P. and Lewis, R. E. (1988) *J. Biol. Chem.* **263**, 11017–11020
- de la Luna, S., Soria, I., Pulido, D., Ortin, J. and Jimenez, A. (1988) *FEBS Lett.* **62**, 121–126
- Dickens, M., Chin, J. E., Roth, R. A., Ellis, L., Denton, R. M. and Tavare, J. M. (1992) *Biochem. J.* **287**, 201–209
- Ebina, Y., Ellis, L., Jarnagin, K., Edery, M., Graf, L., Clauser, E., Ou, J., Masiazar, F., Kan, Y. W., Goldfine, I. D., Roth, R. A. and Rutter, W. J. (1985) *Cell* **40**, 747–758
- Ellis, L., Clauser, E., Morgan, D. O., Edery, M., Roth, R. A. and Rutter, W. J. (1986) *Cell* **45**, 721–732
- Feener, E. P., Backer, J. M., King, G. L., Wilden, P. A., Sun, X. J., Kahn, C. R. and White, M. F. (1993) *J. Biol. Chem.* **268**, 11256–11264
- Grunberger, G. (1991) *Cell Signalling* **3**, 171–177
- Haring, H. U. (1991) *Diabetologia* **34**, 848–861
- Inoguchi, T., Battan, R., Handler, E., Sportsman, R., Heath, W. and King, G. L. (1992) *Proc. Natl. Acad. Sci. U.S.A.* **89**, 11059–11063
- Issad, T., Young, S. W., Tavare, J. M. and Denton, R. M. (1992) *FEBS Lett.* **296**, 41–45
- Jacobs, S. and Cuatrecasas, P. (1986) *J. Biol. Chem.* **261**, 934–939
- Karasik, A., Rothenberg, P. L., Yamada, K., White, M. F. and Kahn, C. R. (1990) *J. Biol. Chem.* **265**, 10226–10231
- Koshio, I., Akanuma, Y. and Kasuga, M. (1989) *FEBS Lett.* **254**, 22–24
- Kunkel, T. A., Roberts, J. D. and Zakour, R. A. (1987) *Methods Enzymol.* **154**, 367–382
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- Lewis, R. E., Caro, L., Perregaux, D. and Czech, M. P. (1990a) *Biochemistry* **29**, 1807–1813
- Lewis, R. E., Wu, G. P., MacDonald, R. G. and Czech, M. P. (1990b) *J. Biol. Chem.* **265**, 947–954
- Luo, K., Hurley, T. R. and Sefton, B. M. (1991) *Methods Enzymol.* **201**, 149–152
- Pillay, T. S., Whittaker, J., Lammers, R., Ullrich, A. and Siddle, K. (1991) *FEBS Lett.* **288**, 206–211
- Rapuano, M. and Rosen, O. M. (1991) *J. Biol. Chem.* **266**, 12902–12907
- Roth, R. A. and Beaudoin, J. (1987) *Diabetes* **36**, 123–126
- Smith, D. M., King, M. J. and Sale, G. J. (1988) *Biochem. J.* **250**, 509–519
- Stadtmauer, L. and Rosen, O. M. (1986) *J. Biol. Chem.* **261**, 3402–3407
- Takata, Y., Webster, N. J. and Olefsky, J. M. (1992) *J. Biol. Chem.* **267**, 9065–9070
- Takayama, S., White, M. F. and Kahn, C. R. (1988) *J. Biol. Chem.* **263**, 3440–3447
- Takebe, Y., Seiki, M., Fujisawa, J., Hoy, P., Yokoto, T., Arai, K., Yoshida, M. and Arai, N. (1988) *Mol. Cell. Biol.* **8**, 466–472
- Tavare, J. M. and Denton, R. M. (1988) *Biochem. J.* **252**, 607–615
- Tavare, J. M., Zhang, B., Ellis, L. and Roth, R. A. (1991) *J. Biol. Chem.* **266**, 21804–21809
- Thies, R. S., Molina, J. M., Ciaraldi, T. P., Freidenberg, G. R. and Olefsky, J. M. (1990) *Diabetes* **39**, 250–259
- Treadway, J. L., Whittaker, J. and Pessin, J. E. (1989) *J. Biol. Chem.* **264**, 15136–15143
- West, M. H. P., Wu, R. S. and Bonner, W. M. (1984) *Electrophoresis* **5**, 133–138
- White, M. F., Livingston, J. N., Backer, J. M., Lauris, V., Dull, T. J., Ullrich, A. and Kahn, C. R. (1988) *Cell* **54**, 641–649
- Wilden, P. A., Siddle, K., Haring, E., Backer, J. M., White, M. F. and Kahn, C. R. (1992) *J. Biol. Chem.* **267**, 13719–13727