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Identification of serines-1035/1037 in the kinase domain of the insulin receptor as protein kinase Cα mediated phosphorylation sites

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Abstract A new site of serine phosphorylation (Ser-1035/1037) has been identified in the kinase domain of the insulin receptor. Mutant receptors missing these two serines were expressed in Chinese hamster ovary cells overexpressing protein kinase Cα. These mutant receptors lacked a phorbol ester-stimulated phosphoserine containing tryptic peptide as demonstrated by both high percentage polyacrylamide/urea gel electrophoresis and two-dimensional tlc. Moreover, a synthetic peptide with the sequence of this tryptic peptide was phosphorylated by isolated protein kinase Cα and co-migrated with the phosphopeptide from in vivo labeled receptor. These results indicate that serine-1035 and/or 1037 in the kinase domain of the insulin receptor are phosphorylated in response to activation of protein kinase Cα.

Key words: Insulin resistance; Tyrosine kinase; Protein kinase C

1. Introduction

Insulin induces extensive tyrosine phosphorylation of the cytoplasmic domain of the β-subunit of its receptor (IR) with at least six tyrosines being phosphorylated, three in the kinase domain, one in the juxtamembrane region and two in the carboxy tail of the receptor [1–3]. This tyrosine phosphorylation appears to greatly activate the intrinsic tyrosine kinase activity of the receptor to phosphorylate various endogenous substrates. Two tyrosine residues in the kinase domain of the receptor (Tyr-1162 and 1163) appear to primarily contribute to this activation of the receptor kinase. In addition to this tyrosine phosphorylation, the IR β-subunit is also extensively phosphorylated on serine residues and to a limited extent on threonine [4,5]. Two serines (Ser-1305/1306) and one threonine (Thr-1348) in the carboxy tail of the receptor have been found to be phosphorylated in vitro by purified protein kinase C (PKC) and in vivo after activation of PKC [6–9]. However, the phosphorylations of these serines and the threonine appear to have little affect on the receptor kinase activity.

The Ser/Thr phosphorylation of the receptor is of potential importance in the negative regulation of the IR tyrosine kinase activity that has been observed in the receptor from many non-insulin-dependent diabetic patients with insulin resistance [10]. It could also be involved in the desensitization of the receptor, either in response to insulin itself or through the activation of other signaling systems by various counter regulatory hormones such as tumor necrosis factor or glucagon [11–13]. An inhibition of the receptor's intrinsic tyrosine kinase activity has been demonstrated in several such systems. In one, Chinese hamster ovary (CHO) cells overexpressing protein kinase Cα and the IR, activation of PKC was shown to greatly increase the serine phosphorylation of the receptor and to inhibit the ability of insulin to stimulate the tyrosine phosphorylation of its endogenous substrate, IR substrate-1 (IRS-1) [14]. In the present studies we have made use of this system to try to identify new serine phosphorylation sites in the IR. One such site that has now been identified (serine-1035 and/or 1037) is in the kinase domain of the receptor, close to a region which is critical for ATP binding.

2. Experimental

2.1. Site-directed mutagenesis and expression of mutant receptors

A 2.4 kb *Bam*HI–*Xba*I fragment isolated from the human IR cDNA [15] was subcloned in to pBluescript II SK plasmid(Stratagene) and used as the template to generate mutant S1035/7A and 1035/7D. Oligonucleotide-directed mutagenesis was carried out as described by Kunkel et al. [16]. Chemically synthesized oligonucleotides used for mutagenesis are as follows (the mismatched bases are underlined): $S^{1035/7A}$ 5′-CGGTCAACGAGGCAGCCGCTCTCCGAGAGC′-(destroy *Ple*I site); $S^{1035/7D}$ 5′-CGGTCAACGAGGATGCCGATCTCCGAGAGC-′ (new *Bsa*BI site). The recombinant plasmid containing the mutant IR cDNA was used to transform $DH5_\alpha$ and the mutations were identified by both restriction enzyme analysis and DNA sequencing. The mutant cDNAs were subcloned into the SR_α expression vector [17] and transfected into CHO/PKCα cells as previously described [18]. Positive colonies were selected by Western with anti-phosphotyrosine antibody RC-20 (Transduction Laboratories) and confirmed by blotting with anti-IR antibodies.

2.2. In vitro phosphorylation of the synthetic peptide

The synthetic peptide, TVNESASLR, corresponding to amino acids 1031 to 1039 deduced from human IR cDNA was phosphorylated by immunoprecipitated protein kinase Cα. The reaction buffer (100 μl) contained 20 mM Tris-HCl, pH 7.5, 20 mM MgCl2, 1 mM $CaCl_2$, 5 μM ATP, 20 μg synthetic peptide, 10 μM TPA, 0.2 mg/ml phosphatidylserine in Triton X-100 mixed micelles, and PKCα bound to anti-PKC monoclonal antibody (Amersham) coated protein G-Sepharose. After 20 min at 24°C, the reaction was terminated by centrifugation and the supernatant was transferred to a fresh tube, treated with activated charcoal, and subjected to high percentage PAGE analysis.

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

Tryptic digestion of ^{32}P-labeled human IR was conducted as described by Luo et al. [19]. In brief, the radiolabled IR was first treated with a tyrosine phosphatase to remove any phosphotyrosine present [18] and then subjected to SDS-PAGE, blotted to nitrocellulose membrane, and digested with 20 μg TPCK-treated trypsin (Worthington) in a freshly prepared 50 mM ammonium bicarbonate buffer at 37°C for 18 h. An additional 10 μg of TPCK-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, and 6 M urea, with Bromophenol blue. High percentage alkaline polyacrylamide gels were prepared according to West et al. [20]. Electrophoresis was carried out overnight at a constant

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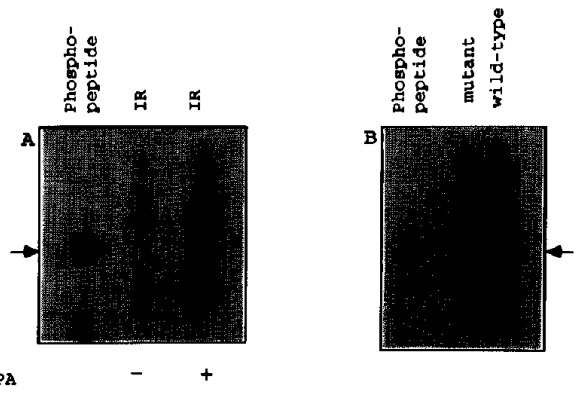

Fig. 1. Autoradiogram of ^{32}P-labeled tryptic peptides separated by high percentage/urea gel electrophoresis. (A) Phosphorylation of a synthetic peptide and co-migration with an IR tryptic phosphopeptide. IR, isolated from in vivo labeled cells treated with or without 1.6 μM TPA, was digested with trypsin as described in the Methods. A synthesized 9-mer peptide was phosphorylated by PKC in vitro. The resulting phosphopeptides were analyzed by high percentage polyacrylamide/urea gel electrophoresis. (B) Comparison of the tryptic phosphopeptides of the wild-type and mutant Ser1035/7A IR. IR was isolated from TPA-treated in vivo labeled cells expressing either the native or mutant IR, digested with trypsin and analyzed by gel electrophoresis.

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 μl water containing 5 mg/ml DNP-lysine and 1 mg/ml xylene cyanol FF and spotted on a TLC plate [21]. First-dimension separation was carried out at pH 3.5 in glacial acetic acid/pyridine/water (10:1:189) [21]. The electrophoresis was conducted at 400 V for 2 h. The second dimensional thin-layer chromatography was carried out for 8–10 h in chromatography buffer containing *n*-butanol/pyridine/glacial acetic acid/water (75:50:15:60) [21]. The plates were dried and autoradiographed.

3. Results and discussion

Tryptic digests of IR from TPA-stimulated CHO cells overexpressing both PKCα and IR (CHO/PKC/IR) indicated that at least 7 phosphoserine containing peptides were generated (Fig. 1A). Attempts to sequence these phosphopeptides were unsuccessful. However, the amino acid compositions for some of these peptides were obtained. The amino acid composition of one of the peptides (Ala, Asp, Thr, Ser, Leu, Val, and Ile in a ratio of 1:2:1:2:1.3:0.8:0.7), matched the amino acid composition of a tryptic fragment corresponding to residues 1031 to 1039 in the IR. This peptide, which has a sequence of TVNESASLR, is quite interesting in that it is very close to the ATP binding region of the receptor. Ser-1037 is also conserved among all the members of the IR family. Finally, Ahn et al. had previously reported that a larger peptide (which encompasses this peptide) from IR phosphorylated in vitro with PKC also contained phosphoserine [9].

A peptide corresponding to the amino acid sequence 1031 to 1039 of the IR was chemically synthesized and checked for its ability to be phosphorylated by PKCα in vitro. This peptide was found to be phosphorylated by PKCα and to co-migrate with one of the in vivo labeled, TPA-stimulated phosphopeptides of the receptor (Fig. 1A). To further test whether the two serines in this region of the IR were phosphorylated, site-directed mutagenesis was used to replace these residues in the IR with either alanine (Ser$^{1035/7A}$) or aspartate (S$^{1035/7D}$). The latter mutant was made on the basis of the hypothesis that aspartate may mimic the affect of phosphorylation at these residues by introducing a negative charge at the site [22]. Stable cell lines overexpressing either wild-type or mutant receptors and PKCα were generated and characterized. Both mutant receptors appeared to be processed normally as judged from the fraction of proreceptor and the size of their β-subunits (Fig. 2A). Lines expressing two different levels of mutant IR Ser-1035/7D were studied (Fig. 2A).

To further test whether serines 1035 and 1037 in the human IR are sites of TPA-stimulated phosphorylation in vivo, phosphopeptide mapping of the wild-type and the mutant (S$^{1035/7A}$) receptors from in vivo labeled TPA-treated cells was carried out. The mutant IR exhibited the same tryptic peptide pattern as the wild-type IR except that one of the phosphopeptide bands was missing (Fig. 1B). The in vitro labeled synthetic peptide migrated to the same location as this missing phosphopeptide band (Fig. 1B). The tryptic peptide patterns of the wild-type and mutant IR were also studied by two-dimensional phosphopeptide mapping. Again, the mutant IR showed the same tryptic pattern as the wild-type IR except that one of the phosphopeptides was missing (Fig. 3B). These results demonstrate that serines-1035 and/or 1037 in the kinase domain of the IR are phosphorylated in TPA-stimulated cells, presumably by PKCα since the peptide containing these residues could also be phosphorylated in vitro by isolated PKCα.

To determine whether the mutations affected the IR tyrosine kinase activity, the receptor from insulin-treated cells was immunoprecipitated and blotted with anti-phosphotyrosine antibodies. The extent of IR autophosphorylation correlated with the amount of each receptor present (Fig. 2B). The ability of these mutant receptors to phosphorylate an exogenous substrate, poly (Glu, Tyr), also appeared not to differ significantly from the wild-type receptor (data not shown). To measure the ability of these different receptors to phosphorylate an endogenous substrate, anti-phosphotyrosine precipitable PI 3-kinase activity was examined in cells expressing the different receptors. Insulin stimulated a comparable increase in this amount of activity in the different cells (legend to Fig. 4). Since prior studies of cells expressing the wild-type receptor have shown that TPA pretreatment caused an inhibition of this response [8], we examined whether the responses mediated via the

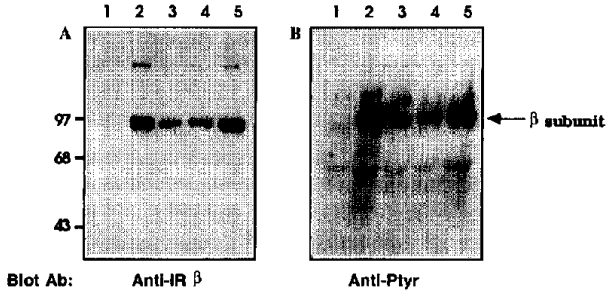

Fig. 2. Expression of the wild-type and mutant IRs. (A) Immunoblotting of the IR with anti-receptor antibody. Anti-IR immunoprecipitates from lysates of insulin-treated CHO/PKC cells transfected with expression vector alone (1), expression vector encoding the wild-type IR (2),or the mutants Ser-1035/7A (3) or Ser1035/7D (4,5) were electrophoresed on 10% polyacrylamide SDS gel, transferred to nitrocellulose and immunoblotted with a monoclonal antibody to the β-subunit of the IR. (B) Anti-phosphotyrosine immunoblotting. The same blot as in panel A was probed with anti-phosphotyrosine antibody RC20.

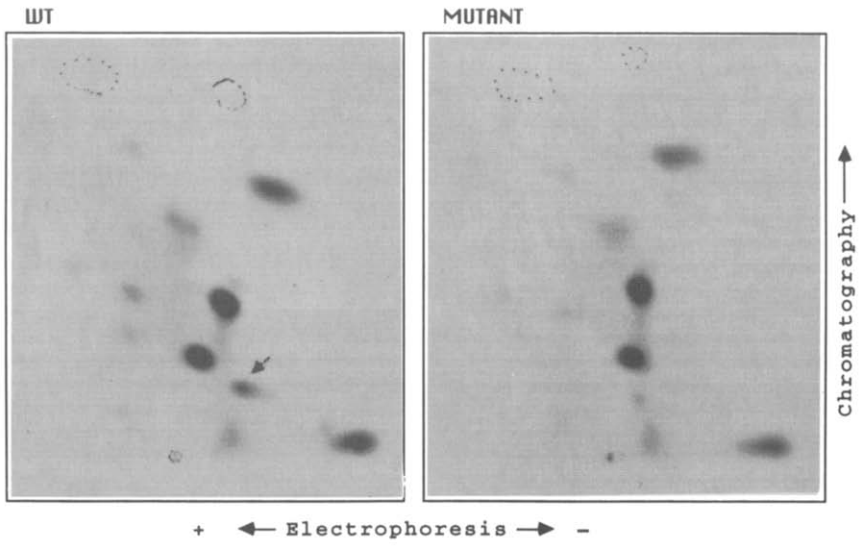

Fig. 3. Comparison of the tryptic phosphopeptides of the wild-type (A) and mutant Ser1035/7A IR (B) by two-dimensional tlc. IR from TPA-stimulated, metabolically labeled cells was precipitated, digested with trypsin and analyzed as described in section 2.

mutant receptors were similarly inhibited. A 60% decrease in the insulin-stimulated response was observed in the cell lines overexpressing either mutant IR 1035/7A or mutant 1035/7D, a value similar to that found for cells overexpressing the wild-type receptor (Fig. 4).

In summary, the present studies have identified the first serine phosphorylation sites in the kinase domain of the insulin receptor. Surprisingly, mutation of these residues to either a neutral amino acid, alanine, or a negatively charged amino acid, aspartate, did not significantly affect the tyrosine kinase activity of the receptor. Moreover, these mutant receptors still exhibited a TPA mediated inhibition of an insulin-stimulated response, the increase in anti-phosphotyrosine precipitable PI 3-kinase activity. These results indicate that the phosphorylation of these serine residues in the kinase domain do not mediate the TPA induced inhibition of this response although it is possible that the phosphorylation of these serines may have other effects on receptor functions.

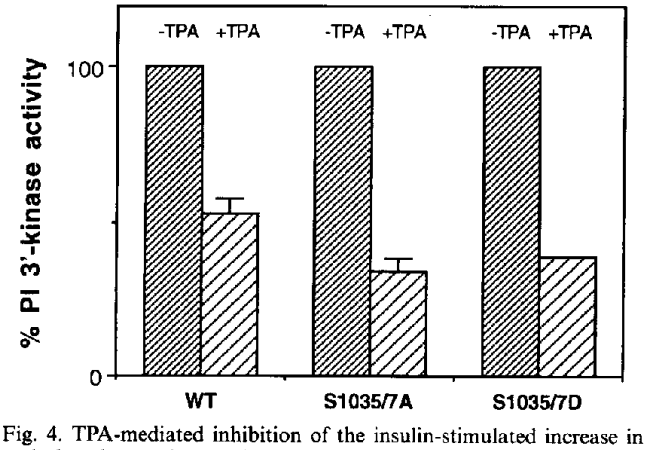

Fig. 4. TPA-mediated inhibition of the insulin-stimulated increase in anti-phosphotyrosine-precipitable PI 3-kinase. Cells expressing wild-type (WT), mutant S1035/7A or mutant S1035/7D IR were treated with insulin in the presence or absence of TPA, lysed and the lysates were adsorbed with anti-phosphotyrosine antibodies. The amount of PI 3-kinase activity in the precipitates was measured and the activity in the absence of TPA was defined as 100%. These values were 1780, 1830 and 2130 for the cells expressing the wild-type, mutant S1035/7A and S1035/7D IR, respectively.

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