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Identification of a phosphorylation site of the rat insulin receptor catalyzed by protein kinase C in an intact cell

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In two-dimensional tryptic phosphopeptide mapping, the β-subunit of the insulin receptor phosphorylated by 12-*O*-tetradecanoylphorbol-13-acetate in rat hepatoma cells (H-35) was separated into one phosphothreonine-containing peptide and several phosphoserine-containing peptides. The synthetic peptide coding residues 1327–1343 in the C-terminal region of the rat insulin receptor was phosphorylated at the threonine residue by protein kinase C in a phosphatidylserine and oleoylacetylglycerol dependent manner. Tryptic digest of this phosphopeptide migrated to the same position as the phosphothreonine containing peptide obtained from the β-subunit in two-dimensional phosphopeptide mapping. These data suggested that Thr 1336 of the insulin receptor is the site of phosphorylation by protein kinase C in intact cells.

Insulin receptor; Protein kinase C; Phosphopeptide mapping, two-dimensional; Phosphothreonine

1. INTRODUCTION

Insulin causes the phosphorylation of tyrosine residues and increases the phosphorylation of serine and threonine residues of its own receptor in an intact cell [1]. Several phosphorylation sites were identified as tyrosine residues 1146 (numbering of amino acids in this paper conforms to that of Ullrich et al. [15]), 1150 and 1151 [2,3]. Tyrosine-phosphorylation of these residues correlates closely with the activation of the insulin receptor-kinase [3,4]. Tyrosine residues at 1316 and 1322 were also reported to be phosphorylated [2,3]. 12-*O*-tetradecanoylphorbol-13-acetate (TPA) increased the phosphorylation of the insulin receptor at serine and threonine residues [5,6]. At the same time, TPA inhibited several insulin effects in intact cell [6]. However, none of the serine- or threonine-phosphorylation sites stimulated by TPA were identified. We show here that TPA stimulates the phosphorylation of threonine residue 1336 in the human sequence of the β-subunit of the insulin receptor in rat hepatoma cell (H-35).

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2. MATERIALS AND METHODS

2.1. *Materials*

The synthetic peptide Gly-Gly-Lys-Lys-Asn-Gly-Arg-Val-Leu-Thr-Leu-Pro-Arg-Ser-Asn-Pro-Ser which codes the carboxy-terminal 17 amino acids of the rat insulin receptor (Lewis, R.E. et al., personal communication) was synthesized by Senshu Biosystems (Tokyo). Autoantibodies to the insulin receptor (B-5 and B-10) were kindly provided by Drs C.R. Kahn (Boston) and S.I. Taylor (Bethesda). Other reagents were purchased from the same sources as described [7] or were of the best commercially available grade.

2.2. *Labeling of the insulin receptor with [^{32}P]orthophosphate*

Rat hepatoma H-35 cells were phosphorylated, solubilized and immunoprecipitated [7]. Immunoprecipitation of the eluate from wheat germ agglutinin-agarose with anti-insulin receptor antibodies was followed by SDS-PAGE as in [7]. Treatment of the cell with 1.6 μM TPA was conducted at 37°C for 30 min.

2.3. *Tryptic phosphopeptide mapping and phosphoamino acid analysis*

The dried polyacrylamide gel containing ^{32}P-labeled insulin receptor β-subunits was cut out, washed and digested sufficient-

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ly with TPCK-trypsin. The digested peptides were analyzed by two-dimensional phosphopeptide mapping [8]. The phosphoamino acid analysis of phosphopeptides was carried out with one-dimensional (pH 3.5) or two-dimensional (pH 1.9 and pH 3.5) thin-layer cellulose electrophoreses [9].

2.4. *Phosphorylation of synthetic peptides of rat insulin receptor by protein kinase C*

Synthetic peptide (0.1 mg) and protein kinase C purified from rat brain as described by House et al. [10] were incubated in 60 μl of 56 mM Tris-Cl buffer (pH 7.4) containing 1 mM DTT, 0.5 mM EDTA, 1.5 mM $CaCl_2$, 3 mM $MgCl_2$, 10 μM [γ-^{32}P]-ATP (5 μCi) with or without 4 μg/ml phosphatidylserine (PtS) and 33 μg/ml 1-oleoyl-2-acetylglycerol (OAG) at 25°C for 10 min. The reaction was terminated by addition of bovine serum albumin and trichloroacetic acid solutions. The supernatant after centrifugation was poured onto phosphocellulose paper. The radioactivity of the paper was measured by Cerenkov counting after washing and drying. The phosphopeptide on the paper was directly digested by TPCK-trypsin as above and purified on a Waters SepPak column [11]. Peptide mapping and phosphoamino acid analysis of the phosphopeptide were carried out as above.

3. RESULTS AND DISCUSSION

The β-subunit of the H-35 rat hepatoma insulin receptor is phosphoprotein. By two-dimensional phosphopeptide mapping, several spots of tryptic phosphopeptides were identified. The assignments and results on the main and clear spots from two-dimensional peptide mapping are shown in fig.1A. The results of phosphoamino acid analysis of these spots are shown in fig.1B. Two phosphoserine-containing peptides (spots b,d) and one weak phosphothreonine-containing peptide (spot a) were clearly identified in the β-subunit of the insulin receptor without stimulation. Additionally, spot c was identified as P_i.

Treatment of H-35 rat hepatoma cell with 1.6 μM TPA for 30 min increased phosphorylation of the insulin receptor β-subunit insulin receptor about 2-fold. In two-dimensional phosphopeptide maps, TPA mainly increased the phosphorylation of the tryptic phosphopeptides designated as spots a, b and d (fig.1A).

Goren et al. [12] demonstrated that mild trypsin digestion (10 μg/ml, for 1–10 min at 22°C) of the wheat germ agglutinin-purified insulin receptor resulted in the loss of the β-subunit C-terminal region containing residues 1316 and 1322. We confirmed that the mildly trypsinized β-subunit (85 kDa) of the insulin receptor obtained from insulin-treated H-35 cells was immunoprecipitated

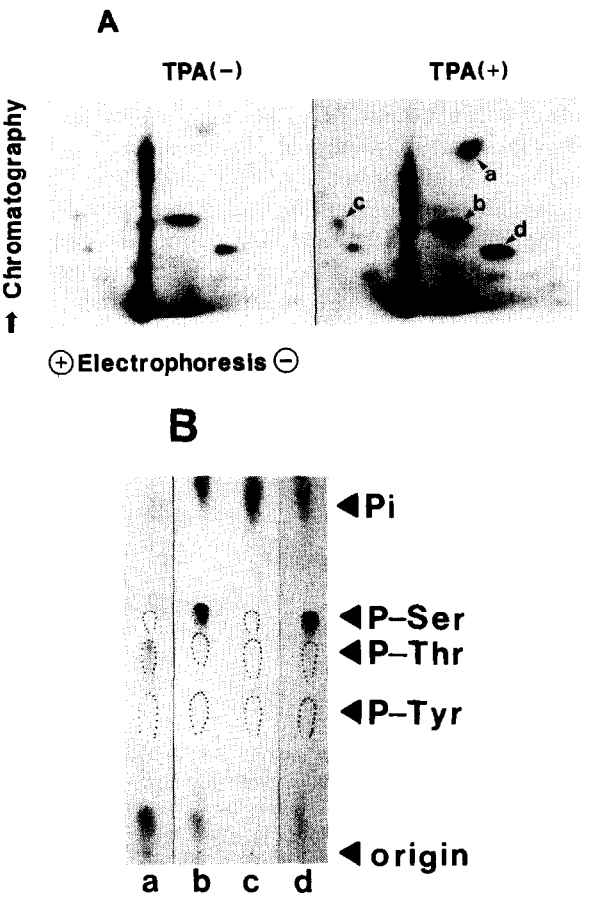

Fig.1. Two-dimensional peptide map of the TPA-stimulated H-35 cell insulin receptor β-subunit and phosphoamino acid identification of its phosphopeptides. (A) Two-dimensional peptide map of the insulin receptor β-subunit obtained from untreated (1) or TPA-stimulated (2) H-35 cells. (B) Phosphoamino acid analysis of spots a–d.

with the antibody specific to kinase domain residues 1142–1153 and autoantibody B-10, but not with the antibody specific to the C-terminal residues 1327–1343 of the insulin receptor (not shown). The same trypsin treatment was conducted followed by two-dimensional phosphopeptide mapping. By this treatment, spots a, b and d were lost in two-dimensional phosphopeptide mapping (not shown) suggesting that these TPA phosphorylated tryptic phosphopeptides were derived from the C-terminal region of the β-subunit. Considering the data that protein kinase C phosphorylates serine or threonine residues located close to basic residues at their N-terminal and/or

C-terminal side [10,13,14], we postulated Thr 1336 and Ser 1340 in the C-terminal region to be the sites of phosphorylation by protein kinase C. The peptide coding residues 1327–1343 was synthesized and phosphorylation attempted with protein kinase C and Ca^{2+} in the presence of PtS and OAG. Phosphorylation of this peptide was increased 3.3-fold in the presence of PtS and OAG. Since the peptide coding residues 1327–1343 contained one threonine and two serine residues, phosphoamino acid analysis was carried out. As shown in fig.2, only phosphothreonine was identified. Next, this phosphopeptide was digested with TPCK-trypsin and separated by two-dimensional thin layer electrophoresis and chromatography. The tryptic digest of the phosphopeptide coding residues 1327–1343 migrated to the same location of spot a in two-dimensional phosphopeptide mapping (fig.3). We concluded that both phosphopeptide 1327–1343 and spot a contained the same tryptic phosphopeptide. These results suggest that TPA phosphorylates Thr 1336 in the human sequence of the rat insulin receptor β-subunit by activating protein kinase C in an intact cell.

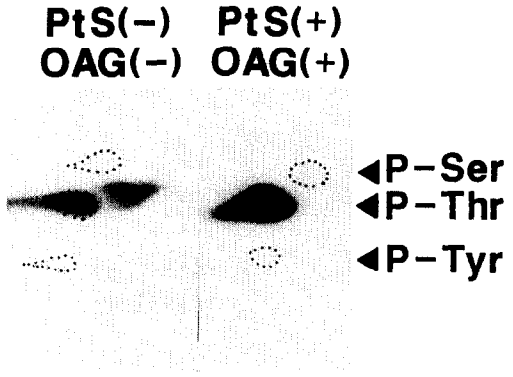

Fig.2. Phosphoamino acid analysis of protein kinase C-catalyzed synthetic C-terminal peptide in the rat insulin receptor. The rat insulin receptor synthetic peptide containing the C-terminal 17 residues was phosphorylated with Ca^{2+} and rat brain purified protein kinase C in the absence (1) and presence (2) of PtS and OAG as described in section. 2. An ambiguous spot shown close to phosphothreonine in the left panel was phosphorylated material which had migrated from another origin.

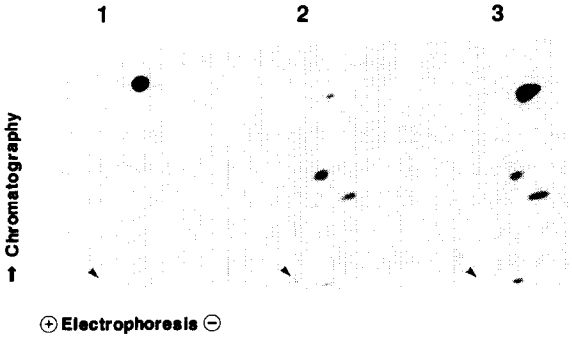

Fig.3. Peptide maps of protein kinase C-catalyzed synthetic C-terminal peptide and phosphorylated insulin receptor. Peptide maps of tryptic peptides derived from protein kinase C-catalyzed synthetic C-terminal peptide in the rat insulin receptor (1), β-subunit of TPA-stimulated H-35 cell insulin receptor (2) and mixture (3) of the peptides shown in panels 1 and 2 are shown as autoradiograms. Origin of electrophoresis is shown by the arrow.

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