Tyrosine phosphorylation of two cytosolic proteins of 50 kDa and 35 kDa in rat liver by insulin-receptor kinase *in vitro*

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Insulin-receptor tyrosine kinase can phosphorylate a variety of artificial substrates *in vitro*. Its physiological substrate(s), however, remains unknown. In the present study, we show that immobilized insulin receptors phosphorylate tyrosine residues of two cytosolic proteins of 50 kDa and 35 kDa in rat liver. Phosphorylation of these two proteins required Mn^{2+} - or Mg^{2+} -ATP as the phosphate donor. Phosphorylation was time- and temperature-dependent. Furthermore, the rate of phosphorylation of the two proteins was related to the autophosphorylated state of the insulin receptor. The pI of the phosphorylated 50 kDa and 35 kDa proteins was 5.4 and 5.6 respectively. These proteins were present in low abundance. They were not related to each other, nor to the insulin receptor, as demonstrated by in-gel proteolytic digestion and by immunoprecipitation using antibodies produced against them. They were specific substrates for the insulin receptor kinase, since they were not phosphorylated by epidermal-growth-factor-receptor kinase. These observations suggest that the 50 kDa and 35 kDa cytosolic proteins may be endogenous substrates for the insulin-receptor kinase.

INTRODUCTION

Insulin initiates its biological actions by binding to its receptor on the cell surface. One of the earliest events after binding is the phosphorylation of tyrosine residues in the β -subunit of the insulin receptor [1–8]. In a cell-free system, it has been demonstrated that receptor autophosphorylation increases the tyrosine phosphotransferase activity towards several artificial substrates [9–14]. In intact cells, inhibition of the receptor kinase [15] or kinase-deficient receptor [16] abolishes the effects of insulin. At present, the biological significance of receptor autophosphorylation and the activation of its kinase activity in the action of insulin is not completely understood [17–19]. It is possible that the activated insulin receptor may initially phosphorylate one or more membrane or cytosolic proteins which catalyse subsequent changes in the activities of key enzymes. Therefore, the identification of such cellular substrate(s) for the insulin receptor kinase would contribute to a better understanding of the mechanism of insulin action. Several putative substrates have been identified. For example, Sadoul *et al.* [20] and Rees-Jones & Taylor [21] demonstrated an insulin-stimulated tyrosine phosphorylation of proteins of 110 kDa and 120 kDa respectively. These two proteins were present in the detergent-solubilized insulin-receptor preparation that had been partially purified by lectin affinity chromatography. White and co-workers [22] identified a phosphotyrosine protein of 185 kDa after the initial binding of insulin to the Fao hepatoma cell line. Haring *et al.* [23] recently reported that insulin stimulated tyrosine phosphorylation of a 46 kDa membrane protein in intact fat-cells. Chou *et al.* [16] showed that insulin stimulates tyrosine phosphorylation of a 150 kDa and 35 kDa protein in Chinese-hamster ovary cell lines. However, the physiological roles of these proteins are not known.

If insulin-receptor tyrosine kinase were indeed to

phosphorylate specific cellular proteins in intact cells, it is likely that these protein substrates would also be phosphorylated by the insulin receptor *in vitro*. In the present study, we used immobilized insulin receptors to probe for the receptor substrate(s) in rat liver cytosolic protein fractions. A purified and concentrated preparation of receptor tyrosine kinase can be prepared by utilizing the high-affinity binding of the insulin receptor to insulin-Sepharose [11]. The immobilized receptor kinase can be activated by autophosphorylation with millimolar concentrations of ATP, and phosphorylation of substrates can be carried out subsequently in micromolar ATP. Using this immobilized enzyme, we have studied the phosphorylation of rat liver cytosolic proteins.

MATERIALS AND METHODS

Materials

Insulin was obtained from Connaught Laboratories, Toronto, Canada. [^{32}P]ATP and [^{32}P]P_i were purchased from ICN Biomedicals Canada Ltd. Triton X-100, phenylmethanesulphonyl fluoride, leupeptin, pepstatin and aprotinin were from Sigma Chemical Co., St. Louis, MO, U.S.A. Reagents for SDS/polyacrylamide-gel electrophoresis were from Bio-Rad Laboratories (Canada) Ltd., Mississauga, Canada. Anti-phosphotyrosine antibody conjugated to Sepharose (2G8-4B) was generously given by Dr. R. Frackelton (Department of Medicine, Brown University, Providence, RI, USA). EGF (epidermal growth factor) and EGF receptor were kindly supplied by Dr. J. Kudlow (Department of Clinical Biochemistry, University of Toronto).

Preparation of immobilized insulin receptor

Rat livers (10 g) were homogenized in 25 ml of 10 mM-Tris/HCl, pH 7.4, containing 5 mM-EDTA, 10 % (v/v)

Fig. 1. Phosphorylation of the hepatic cytosolic proteins by insulin-receptor–Sepharose

A 2 ml portion of rat liver supernatant was fractionated on a column of Sephadex G-200 as described in the text. A 40 μ l sample from alternate fractions as indicated was incubated with Mn- 32 P]ATP in the absence (–) or presence (+) of activated receptor–Sepharose (P-InR). The reaction mixture was analysed by SDS/polyacrylamide-gel electrophoresis and autoradiography. The phosphorylated 50 kDa and 35 kDa bands are identified, respectively, by black and white arrowheads. Positions of molecular-mass markers in kDa are indicated.

glycerol, 10 mM-aminohexanoic acid, 10 mM-benzamidine, 0.25 M-sucrose, 100 μ M-phenylmethanesulphonyl fluoride, bacitracin (1 mg/ml), leupeptin (1 μ g/ml), pepstatin (1 μ g/ml) and aprotinin (1 μ g/ml). The preparation of microsomal membranes and the solubilization of the insulin receptor in Triton X-100 were carried out as described previously [11]. The insulin–Sepharose was prepared by reaction of insulin with succinyl-diaminodipropylamine–Sepharose (1 mg of insulin/ml of settled beads) by the method of Cuatrecasas & Parikh [24]. Binding of the solubilized insulin receptor to insulin–Sepharose was carried out at 4 °C for 16 h. The receptor–insulin–Sepharose was washed with 50 ml of 50 mM-Tris/HCl, pH 7.4, containing 0.2 % Triton X-100 and 1 M-NaCl, and then with 50 ml of the same buffer without NaCl. Where indicated, the immobilized insulin receptor was phosphorylated with 2 mM-ATP in the presence of 10 mM-MnCl₂ at 24 °C for 30 min, washed and then used.

Preparation and phosphorylation of cytosolic substrates

A 2 ml sample of liver cytosol, obtained as the supernatant from the preparation of microsomal membranes as described above, was fractionated at 4 °C on a column (1.5 cm $\times$ 90 cm) of Sephadex G-200 equilibrated with 50 mM-Tris/HCl, pH 7.4: 3 ml fractions were collected and stored at –76 °C. Phosphorylation was carried out at 24 °C for 15 min in a reaction mixture (80 μ l) containing 20 μ l of insulin-receptor–Sepharose, 40 μ l of each fraction, 10 mM-MnCl₂ or -MgCl₂, 5 μ M-ATP (containing 5 μ Ci of 32 P]ATP), 50 mM-Tris/HCl, pH 7.4, and 0.5 % Triton X-100 for 15 min. The reaction was terminated by the addition of 20 μ l of a solution containing 0.45 M-dithiothreitol, 60 mM-EDTA, 6 % (v/v) SDS and 30 % (w/v) sucrose. The reaction mixture was subjected to SDS/polyacrylamide-gel

electrophoresis in a 10 % acrylamide gel. The gel was stained [25] and dried. The radioactive bands located by autoradiography were excised and their radioactivity was determined by scintillation counting.

Phosphoamino acid analysis

The phosphoprotein bands were excised from the gel and homogenized in 1 ml of 0.5 % SDS. The phosphoproteins were co-precipitated with 1 mg of bovine serum albumin by adding 5 vol. of acetone at –20 °C. The precipitates were washed with cold acetone, dried, and hydrolysed in 0.5 ml of 6 M-HCl for 2 h at 110 °C *in vacuo*. The phosphoamino acids were separated by two-dimensional thin-layer electrophoresis at pH 1.9 and pH 3.5, and analysed by autoradiography [4]. Phosphoamino acid standards were added to the hydrolysed samples and were located by ninhydrin staining.

Antibody production and immunoprecipitation

Polyclonal antibodies against phosphotyrosine were prepared as described by Pang *et al.* [26]. Antibodies against the 50 kDa and 35 kDa proteins were prepared in rabbits. The proteins were electroeluted from the SDS/polyacrylamide-gel-electrophoresis gel. The animals were immunized three times with the proteins in complete Freund's adjuvant at intervals of 10–14 days and then once with the proteins in incomplete Freund's adjuvant. Immunoprecipitation was carried out by incubating 100 μ l of the phosphorylation reaction mixture with 10 μ l of immune serum at 4 °C for 16–20 h. The immune complex, precipitated by Protein A–Sepharose (20 μ l of settled beads) at 4 °C for 2 h, was solubilized by heating for 5 min in 40 μ l of a solution containing 250 mM-dithiothreitol, 30 mM-EDTA, 3 % SDS and 15 % sucrose, and then analysed by SDS/polyacrylamide-gel electrophoresis and autoradiography.

Fig. 2. Time course of the phosphorylation of the 50 kDa and 35 kDa bands by insulin-receptor beads not previously activated with ATP (InR) or by ATP-activated insulin-receptor-Sepharose (P-InR)

Samples (40 μ l) from the column fractions containing the 50 kDa or 35 kDa proteins were incubated with 5 μ M-ATP and 10 mM-MnCl₂ at 24 °C for the periods indicated. Positions of the phosphorylated bands of the receptor β subunit (95 kDa) and the 50 kDa and 35 kDa substrates are indicated.

Fig. 3. Effect of temperature on the phosphorylation of the 50 kDa and 35 kDa proteins by activated insulin-receptor-Sepharose

Phosphorylation was carried out at either 24 °C (○) or 4 °C (●) in the presence of 5 μ M-ATP and 10 mM-MnCl₂.

Fig. 4. ATP requirement for the phosphorylation of the 50 kDa and 35 kDa proteins in the presence of the activated (○) and non-activated (●) insulin-receptor-Sepharose

Phosphorylation was carried out in the presence of various concentrations of ATP and 10 mM-MnCl₂ for 15 min at 24 °C.

Fig. 5. Identification of [^{32}P]phosphoamino acids in the phosphorylated 50 kDa and 35 kDa bands by two-dimensional thin-layer electrophoresis and autoradiography

O indicates the origin of sample application. Panels (b) and (d) are autoradiographic analyses of the 50 kDa and 35 kDa band respectively. Panels (a) and (c) are the respective ninhydrin-stained patterns of added phosphoamino acid standards.

RESULTS

Fig. 1 shows the insulin-receptor-catalysed phosphorylation of liver cytosolic proteins in fractions obtained from gel filtration of soluble proteins on Sephadex G-200. The phosphorylation of protein bands (95 kDa, 50 kDa and 35 kDa) was dependent on the addition of immobilized insulin receptors. A phosphorylated 95 kDa band, present in all the fractions incubated with the insulin-receptor beads, was probably the β subunit receptor. A 50 kDa band in fractions 23–27 and a 35 kDa band in fractions 31–39 were also phosphorylated by the insulin receptor beads. In fractions 35–39, a doublet of 35 kDa was evident. The electrophoretic mobility of the 50 kDa and the 35 kDa bands remained unchanged after reduction with dithiothreitol. This observation suggests that they were not disulphide-linked to other components. The gel-filtration fractions containing the 50 kDa protein were collected at an elution volume slightly ahead of bovine serum albumin, suggesting that its molecular mass in Sephadex was greater than 67 kDa. It would appear that under non-denaturing conditions the 50 kDa protein either self-aggregated or was associated with other cytosolic components.

The rate of phosphorylation of the 50 kDa and 35 kDa proteins was affected by the phosphorylation state of the immobilized insulin receptor. As shown in Fig. 2, insulin receptors (P-InR) which had been activated with ATP increased the rate of phosphorylation of these two

Fig. 6. Binding of the 50 kDa and 30 kDa phosphoproteins to monoclonal antiphosphotyrosine antibody

After the phosphorylation by the insulin-receptor kinase, the supernatant containing the 50 kDa protein (lane a) and the 35 kDa protein (lane d) were incubated with the monoclonal antiphosphotyrosine antibody conjugated to Sepharose (2G8-S4B) for 16 h at 4 °C. The unbound phosphoproteins in the 50 kDa (b) and 35 kDa (e) mixtures were removed. After washing the beads, the bound 50 kDa (c) and 35 kDa (f) proteins were eluted with 50 mM-phenyl phosphate at 4 °C for 30 min. Positions of the 50 kDa and 35 kDa bands are indicated.

Fig. 7. Proteolytic in-gel digestion of the 95 kDa, 50 kDa and 35 kDa phosphoproteins

Gel pieces containing the 95 kDa (lanes *a*, *b* and *c*), 50 kDa (lanes *d*, *e* and *f*) and 35 kDa (lanes *g*, *h* and *i*) phosphoproteins were digested with 20 μ g of chymotrypsin (*b*, *e* and *h*) or *Staphylococcus aureus* proteinase (*c*, *f* and *i*). Lanes *a*, *d* and *g* were controls without enzyme digestion. The positions of the molecular-mass markers in kDa are indicated.

bands, compared with the non-activated insulin receptor (InR). Phosphorylation of the 50 kDa band was evident after only 1 min at 24 °C in the presence of the activated insulin-receptor beads, and the phosphorylation of both the 50 kDa and the 35 kDa bands appeared to have reached a plateau after 30 min (results not shown). The rate of phosphorylation of the proteins was temperature-dependent (Fig. 3). The effect of activation of the receptor with ATP on the phosphorylation of the two bands is further demonstrated by the data presented in Fig. 4. Activated receptors required a concentration of ATP of 5–10 μ M in the incubation mixture to achieve a maximal phosphorylation of the bands. In contrast, receptors without prior activation with ATP required more than 10 μ M-ATP for maximal phosphorylation, and at 2 μ M-ATP phosphorylation of the two bands was not observed. These results are consistent with the previous observation that autophosphorylation of the insulin receptor enhanced its kinase activity [14]. The apparent affinity of the immobilized insulin receptor for MnATP in the phosphorylation reaction of these proteins is in the micromolar range (Fig. 4). MgATP can substitute for MnATP, but with a lower apparent affinity.

The radioactivity incorporated into the 50 kDa and 35 kDa bands was resistant to 1 M-KOH at 55 °C for 2 h [27], suggesting that incorporation was on the tyrosine residues. Phosphoamino acid analysis of the phosphorylated 50 kDa and 35 kDa bands shows that, for both proteins, phosphorylation occurred on tyrosine residues (Fig. 5). Monoclonal and polyclonal antibodies, directed against phosphotyrosine, were able to immunoprecipitate these two proteins (Fig. 6, lanes *c* and *f*, and Fig. 8, lanes *b* and *f*). These results demonstrate that the immobilized insulin receptor phosphorylated the tyrosine residues of

these cytosolic proteins. The pI of the phosphorylated 50 kDa proteins was determined to be 5.4 by isoelectrofocusing. The 35 kDa doublet was further resolved into multiple spots with pI 5.5–5.6.

We considered the possibility that the 50 kDa and 35 kDa proteins could be derived from the 95 kDa subunit of the insulin receptor. Proteolytic cleavage of the gel bands of the phosphorylated 95 kDa, 50 kDa and 35 kDa proteins with chymotrypsin or V8 protease [28] yielded very different phosphopeptide patterns (Fig. 7). Antibodies obtained from a rabbit immunized against the 50 kDa protein were able to precipitate the phosphorylated 50 kDa protein, but not the receptor β subunit (Fig. 8, lane *c*) nor the phosphorylated 35 kDa protein (Fig. 8, lane *g*). Similarly, antibodies prepared against the 35 kDa protein were specific for the phosphorylated 35 kDa protein (Fig. 8, lanes *d* and *h*). Taken together, these results demonstrate that the 95 kDa, 50 kDa and 35 kDa proteins were unrelated to each other in primary structure, and they are not associated with each other as an immunoprecipitable complex.

The phosphorylated 50 kDa band and the 35 kDa doublet did not correspond to any major silver-stained or Coomassie-Blue-stained bands on the gel. Assuming the incorporation of phosphate to be 1 mol/mol, we estimate that there are about 100 000 molecules of each substrate per cell or 1 nmol of each substrate per 15 g of rat liver.

These two hepatic proteins appear to be specific substrates for insulin-receptor kinase, since EGF receptors prepared from A431 cell lines did not stimulate the phosphorylation of the two proteins under conditions optimal for EGF-receptor kinase activity at 4 °C (Fig. 9) or at 24 °C (results not shown). Insulin-receptor-

Fig. 8. Immunoprecipitation of the 95 kDa, 50 kDa and 35 kDa phosphoproteins by polyclonal antibodies against phosphotyrosine, 50 kDa protein and 35 kDa protein

After phosphorylation of the 50 kDa and 35 kDa proteins by the insulin-receptor-Sepharose, the reaction mixtures were frozen and thawed several times to dissociate the phosphorylated insulin receptor from insulin-Sepharose. The supernatants containing the phosphorylated 50 kDa protein (lanes *a*, *b*, *c* and *d*) or the phosphorylated 35 kDa protein (lanes *e*, *f*, *g* and *h*) were precipitated with normal serum (*a*, *e*), polyclonal anti-phosphotyrosine antibodies (*b*, *f*), anti-50 kDa-protein antibodies (*c*, *g*) and anti-35 kDa-protein antibodies (*d*, *h*). The phosphorylated receptor β subunit of 95 kDa present in the supernatant was also precipitated by the anti-phosphotyrosine antibodies (*b* and *f*).

catalysed phosphorylation of these proteins seems to be unique to the liver, since we have not been able to demonstrate the phosphorylation of the two proteins in fractionated cytosol from brain, heart and adipose tissues of the rat. Under similar conditions, activated insulin receptors catalysed the phosphorylation of a 50 kDa and 35 kDa protein in the fractionated cytosols from livers of rabbit and mouse (results not shown).

DISCUSSION

In this study we observed that immobilized insulin receptor catalysed the phosphorylation of two hepatic proteins of 50 kDa and 35 kDa *in vitro*. The phosphorylation of these proteins was exclusively on tyrosine residues, as determined by phosphoamino acid analysis (Fig. 5). Phosphorylation was time- and temperature-dependent and saturable. It required micromolar concentrations of ATP and was enhanced when the insulin receptor was previously activated by autophosphorylation (Fig. 2). These characteristics suggest that phosphorylation of these proteins may be involved in the action of insulin. Phosphorylation of these proteins could be demonstrated only after fractionation by gel filtration or ion-exchange chromatography, but not in the initial unfractionated cytosol. Furthermore, when the unfractionated liver cytosol was incubated with the

Fig. 9. Inability of EGF-receptor tyrosine kinase to phosphorylate the rat liver cytosol 50 kDa and 35 kDa proteins

Plasma membranes prepared from A431 cells were incubated with (+) or without (-) EGF (0.1 μ M) for 10 min at 4 $^{\circ}$ C in 20 mM-Hepes buffer, pH 7.4. The phosphorylation reaction was carried out in 20 mM-MgCl₂, 1 mM-CaCl₂, 10 μ M-sodium orthovanadate and 0.5 μ M-ATP for 10 min at 4 $^{\circ}$ C. Fractions containing the 50 kDa or 35 kDa protein were added as indicated. The phosphorylated 170 kDa EGF receptor band is indicated by the arrowhead. The phosphorylation of the endogenous substrate for the EGF-receptor kinase is also indicated (●).

immobilized insulin receptor, phosphorylation of the β subunit of the receptor was not observed. Fractionation may have removed or decreased the amount of phosphatase, ATPase or kinase inhibitor(s) which interfered with the phosphorylation reaction.

The identities and functions of these substrates are not known. Results obtained from the in-gel digestion and immunoprecipitation of the two phosphorylated proteins show that they are not derived from β subunit of the insulin receptor nor are they related to each other. In terms of molecular mass, they are also evidently different from the 110 kDa phosphoprotein reported by Sadoul *et al.* [20], the 120 kDa phosphoprotein obtained by Rees-Jones & Taylor [21] and the 180 kDa phosphoprotein detected in Fao hepatoma cells by White *et al.* [22]. Recently Haring *et al.* [23] showed that insulin stimulated the phosphorylation of several protein bands between 45 and 50 kDa in the fat-cell. Chou *et al.* [16] showed that insulin stimulated the phosphorylation of a 150 kDa and a 35 kDa protein. The roles of these phosphoproteins in the action of insulin remain to be established.

It is possible that the liver 35 kDa protein is related to lipocortin and is associated with the cell membrane cytoskeleton [27]. However, the liver 50 kDa and 35 kDa proteins were not phosphorylated by EGF-receptor

kinase. Furthermore, insulin-receptor kinase did not phosphorylate EGF receptor substrate, but phosphorylated the liver substrates in a Ca^{2+} -independent manner. Indeed, Pike *et al.* [10] have previously demonstrated that EGF-receptor kinase and insulin-receptor kinase have similar, but not identical, specificities for peptide substrates and bivalent-metal-ion requirements. Klein and co-workers [29], using exogenous substrates and lectin-purified solubilized rat liver membranes, found that one group of substrates was phosphorylated primarily by insulin-stimulated kinase, and a different group primarily by EGF-stimulated kinase. Insulin receptor and EGF receptor are both growth-factor receptors, and their kinase domains are similar [30,31]. However, insulin is different from EGF in its metabolic effects on the liver. It is therefore reasonable to suggest that transduction of hormonal signals may involve the phosphorylation of substrates that are receptor-specific.

In conclusion, we have detected two liver proteins which appear to be unique substrates for the tyrosine-specific insulin-receptor kinase. These findings further support the idea that insulin receptor phosphorylates other cellular proteins, whose functions are thereby altered. Since the substrate preparations employed in this study were crude, we cannot rule out the possibility that insulin receptor activates other tyrosine kinases, which phosphorylate the liver proteins. Further purification of the 50 kDa and 35 kDa proteins is needed in order to rule out this possibility. The subcellular localization, the identity and the function of these proteins remain unknown. With the availability of the antibodies against these two proteins, it will be possible to demonstrate the insulin-stimulated phosphorylation of these proteins in intact cells and to study their roles in the mechanism of insulin action.

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