Identification of a cellular 110000-Da protein substrate for the insulin-receptor kinase

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Addition of insulin to wheat-germ-lectin-purified glycoproteins derived from rat hepatocytes or rabbit brown adipose tissue results in the increased phosphorylation of a M_r -110000 protein. This naturally occurring glycoprotein appears as a monomeric structure and is not part of the insulin receptor itself, since it is not immunoprecipitated by highly specific antibodies to insulin receptor. Phosphorylation of the M_r -110000 protein and autophosphorylation of the receptor β -subunit (M_r 95000) are stimulated by insulin in a remarkably similar dose-dependent fashion, with half-maximal stimulation at 1 nM-insulin. Further, kinetic studies suggest that the phosphorylation of the M_r -110000 protein occurs after autophosphorylation of the insulin-receptor kinase. In conclusion, the present identification of an endogenous substrate for the insulin-receptor kinase could suggest that some, if not all, effects of insulin may be mediated through activation of this kinase.

Insulin binding to its receptor initiates a large array of biological responses. The mechanism whereby insulin elicits these responses is unknown (Kahn *et al.*, 1981; Denton *et al.*, 1981). In attempts to approach this mechanism, the ability of insulin to promote the phosphorylation or dephosphorylation of cell proteins has been studied for several years (for review, see Denton *et al.*, 1981; Cohen, 1982). Increased interest in this hypothesis was subsequently emphasized with the discovery that insulin enhances the phosphorylation of its own receptor both in whole cells and in cell-free systems (Kasuga *et al.*, 1982*a,b*; Van Obberghen & Kowalski, 1982; Avruch *et al.*, 1982; Petruzelli *et al.*, 1982; Roth & Cassel, 1983; Shia & Pilch, 1983; Gammeltoft *et al.*, 1984). The identification of an ATP-binding site on the receptor β -subunit (Roth & Cassel, 1983; Shia & Pilch, 1983; Van Obberghen *et al.*, 1983), and the demonstration that insulin-sensitive protein kinase activity is found in highly purified insulin receptors (Kasuga *et al.*, 1983; Van Obberghen *et al.*, 1983; Nemenoff *et al.*, 1984; Petruzelli *et al.*, 1984), have led to the conclusion that the insulin receptor is itself an insulin-responsive protein kinase. This enzyme phosphorylates the β -subunit of the receptor as well as a

variety of exogenous substrates (Gazzano *et al.*, 1983; Stadtmauer & Rosen, 1983; Rosen *et al.*, 1983; Zick *et al.*, 1983; Braun *et al.*, 1984). Although the precise physiological significance of this reaction is not established, this very early biochemical event after hormone binding has been postulated to play a crucial role in transmembrane hormone signalling. It is indeed possible that some or all insulin actions may be transferred from the insulin receptor through activation of the receptor kinase system. To establish the validity of this contention, it is essential to show that the insulin-activated receptor kinase can mediate phosphorylation of some other cellular proteins.

In the present study we have identified a 110 kDa cellular protein, which appeared to serve as a natural substrate for the insulin-receptor kinase in a cell extract derived from rat hepatocytes and rabbit brown adipose tissue.

Materials and methods

Chemicals

Pig insulin (monocomponent) was purchased from Novo (Copenhagen, Denmark); collagenase was from Boehringer-Mannheim; Triton X-100, ATP, *N*-acetyl-D-glucosamine, wheat-germ-agglutinin-agarose, bovine serum albumin (fraction V), bacitracin and phenylmethanesulphonyl fluoride

Abbreviation used: SDS, sodium dodecyl sulphate.

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were from Sigma. Aprotinin was from Specia (Paris, France). [γ - 32 P]ATP (triethylammonium salt; aqueous solution; 5000 Ci/mmol) was from The Radiochemical Centre (Amersham, Bucks., U.K.). All reagents for SDS/polyacrylamide-gel electrophoresis were purchased from Bio-Rad. Serum from patient B-9 with antibodies to the insulin receptor was generously given by C. R. Kahn (Joslin Research Laboratory, Boston, MA, U.S.A.).

Animals

Male Wistar rats (120–150 g) were maintained in a constant-temperature (22°C) animal room with free access to water and to a standard laboratory chow. Rabbit foetuses were obtained by caesarian section on day 30 of gestation.

Preparation of partially purified insulin receptors from rat hepatocytes

Rat hepatocytes were isolated by collagenase dissociation as previously described (Le Cam & Freychet, 1977). Immediately after isolation, hepatocytes were washed twice with cold buffer containing Hepes (50 mM, pH 7.6), NaCl (150 mM), bacitracin (1 mM), aprotinin (1000 trypsin-inhibitor units/ml) and benzamidine (2.5 mM). Cells were collected by centrifugation, resuspended in the washing buffer containing Triton X-100 (1%) and homogenized in a Dounce homogenizer (100 strokes). The broken cells were solubilized for 90 min at 4°C with continuous stirring. The cell extract was centrifuged at 150 000 *g* for 90 min at 4°C and the insoluble fraction was discarded. The supernatant was applied to a wheat-germ-agglutinin-agarose column, which was extensively washed at 4°C; bound glycoproteins were desorbed with *N*-acetyl-D-glucosamine (0.3 M), and the fractions which are known to contain insulin receptors (Van Obberghen *et al.*, 1981) were pooled and stored at -20°C without appreciable loss of insulin binding and kinase activity for 1 month.

Preparation of partially purified insulin receptors from rabbit brown adipose tissue

Interscapular brown adipose tissue was homogenized with a Polytron homogenizer (setting 6–7) for 30 s in 5 vol. of ice-cold Hepes/NaCl buffer containing proteinase inhibitors. Thereafter, these homogenates were processed in the same way as the rat hepatocyte homogenates.

Phosphorylation of partially purified insulin receptors

Samples of wheat-germ eluate were incubated in a reaction mixture containing Hepes (50 mM, pH 7.6), NaCl (150 mM), MnCl₂ (4 mM), MgCl₂ (8 mM), proteinase inhibitors, bovine serum albumin (0.1%), and if present, insulin (at the concen-

trations indicated) for 90 min at 20°C to allow insulin binding to occur. Thereafter the phosphorylation reaction was initiated by adding [γ - 32 P]ATP (20 μ M), and the reaction tubes were incubated at 20°C for 15 min or the times indicated. The reaction was terminated by adding either a boiling SDS solution or an ice-cold stopping solution containing NaF and EDTA (final concns. 100 mM and 20 mM respectively).

Immunoprecipitation of phosphorylated insulin receptors

Phosphorylated insulin receptors were specifically immunoprecipitated with antiserum containing autoantibodies to the insulin receptor as described previously (Van Obberghen *et al.*, 1981; Kasuga *et al.*, 1981). In control experiments pooled non-immune sera were used instead of antiserum. The immunoprecipitation was carried out overnight at 4°C and was achieved by addition of *Staphylococcus aureus* cells (Pansorbin). After 4 h at 4°C the immunoprecipitates were collected by centrifugation and washed three times with Hepes/NaCl buffer.

Gel electrophoresis and autoradiography

All samples were solubilized and boiled for 5 min in a solution containing 3% (w/v) SDS, 10% (v/v) glycerol, 10 mM-sodium phosphate, 0.05% Bromophenol Blue (non-reducing conditions) and 5% (v/v) 2-mercaptoethanol (reducing conditions). Samples were analysed by one-dimensional SDS/polyacrylamide-gel electrophoresis as described by Laemmli (1970), with a 7.5% or 5% acrylamide resolving gel. *M_r* values of the standards used were: myosin, 200 000; β -galactosidase, 116 000; phosphorylase *b*, 94 000; bovine serum albumin, 67 000; ovalbumin 43 000; carbonic anhydrase, 30 000; soya-bean trypsin inhibitor, 20 000; lysozyme, 14 000. The gels were processed as described by Van Obberghen *et al.* (1981). The autoradiograms were scanned in a microdensitometer (Gelman) for quantitative analysis. Phosphoamino acid analysis was performed as described by Hunter & Sefton (1980).

Results

Detection of an endogenous protein substrate for the insulin-receptor kinase in cell extracts from rat liver and rabbit brown adipose tissue

We first investigated whether partially purified insulin receptors obtained by wheat-germ-lectin chromatography were able to phosphorylate endogenous proteins. To this end, samples of the lectin-purified glycoproteins isolated from rat liver were incubated without or with insulin (1 μ M) and subjected to the standard phosphorylation procedure.

The phosphoproteins were subsequently analysed by SDS/polyacrylamide-gel electrophoresis followed by autoradiography. When the phosphorylation was performed without preincubation with insulin, a few minor phosphoproteins were observed, ranging in molecular mass from 130 to 40 kDa (Fig. 1, lane *a*). Incubation with insulin ($1 \mu\text{M}$) resulted in the enhancement of the phosphorylation of the receptor β -subunit, as we previously reported (Van Obberghen & Kowalski, 1982; Van Obberghen *et al.*, 1983), but also the appearance of a phosphorylated protein of apparent M_r 110 000 (lane *b*). To characterize the 110 kDa protein further, the phosphoproteins obtained after preincubation in the absence or presence of insulin were analysed by SDS/polyacrylamide-gel electrophoresis under non-reducing conditions. Comparison of lanes (*c*) and (*d*) (Fig. 1) showed that insulin not only enhanced phosphorylation of the insulin-receptor oligomers ($\alpha_2\beta_2$, $\alpha_2\beta$) and of free 95 kDa β -subunits, but also induced a significant increase in the amount of ^{32}P incorporated in the 110 kDa protein. It thus appears that the 110 kDa protein has a monomeric structure. In an attempt to assess whether the 110 kDa endogenous substrate was tissue- or species-specific, we searched for a similar protein in a cell extract prepared from rabbit brown adipose tissue. As shown in Fig. 1 (lanes *e* and *f*), as observed with hepatocytes, addition of insulin led to both autophosphorylation of the re-

ceptor β -subunit and increased labelling of a 110 kDa protein.

Finally, to exclude the possibility that this 110 kDa protein corresponds to the receptor α -subunit, the wheat-germ-purified and phosphorylated glycoproteins were exposed to normal serum or highly specific antibodies to the insulin receptor. SDS/polyacrylamide-gel electrophoresis analysis of the immunoprecipitates revealed that the anti-receptor antibodies immunoprecipitated a 95 kDa phosphoprotein corresponding to the insulin-receptor β -subunit, but failed to immunoprecipitate the 110 kDa phosphoprotein (Fig. 2, lane *b*). Conversely, the 110 kDa phosphoprotein was found in the supernatants obtained after immunoprecipitation with normal serum (Fig. 2, lane *c*) or with anti-receptor antibodies (Fig. 2, lane *d*). To identify the nature of the phospho-amino acid(s) present in the 110 kDa protein, the gel region comprising this peptide phosphorylated after exposure to insulin was excised and processed for phospho-amino acid analysis (Hunter & Sefton, 1980), which revealed the presence of phosphotyrosine (30%), phosphothreonine (55%) and phosphoserine (15%) (results not shown).

Insulin stimulates phosphorylation of the 110 kDa protein in a dose- and time-dependent fashion

To study the time-dependency of phosphorylation, purified glycoproteins were first incubated

Fig. 1. Identification of a 110 kDa endogenous phosphoprotein in cell extracts derived from rat hepatocytes and rabbit brown adipose tissue

Samples of wheat-germ-lectin-purified glycoproteins obtained from rat hepatocytes (lanes *a*–*d*) or rabbit brown adipose tissue (lanes *e* and *f*) were incubated for 90 min at 20°C without or with insulin ($1 \mu\text{M}$), and used in a cell-free phosphorylation assay (15 min at 20°C). The reaction was stopped by addition of boiling SDS solution, and the samples were analysed by SDS/polyacrylamide-gel electrophoresis under reducing conditions (lanes *a*, *b*, *e*, *f*) or non-reducing conditions (lanes *c* and *d*) followed by autoradiography. Key, OR, origin; pp 110, 110 kDa phosphoprotein; β , receptor β -subunit. Numbers to the left of gels are M_r values ($\times 10^{-3}$) of standards.

Fig. 2. The 110 kDa phosphoprotein derived from rat liver is not immunoprecipitated by antibodies to insulin receptor

Samples of wheat-germ-purified glycoproteins obtained from rat hepatocytes were incubated for 90 min at 20°C with insulin (1 μ M) and used in a cell-free phosphorylation assay (15 min at 20°C). The reaction was stopped by adding an ice-cold stopping solution (NaF, 100 mM; EDTA, 20 mM), and thereafter the samples were exposed to normal serum (lanes a, c) or to serum containing autoantibodies to the insulin receptor (patient B-9; Anti-R) at a 1:300 dilution (lanes b, d). Immunoprecipitation was achieved by addition of protein A. The immunoprecipitates (lanes a, b) and their supernatants (lanes c, d) were analysed by SDS/polyacrylamide-gel electrophoresis under reducing conditions, followed by autoradiography. For key see Fig. 1 legend.

with insulin (1 μ M) for 90 min, and thereafter [γ - 32 P]ATP was added to initiate the phosphorylation reaction. At different time intervals the reaction was stopped, and the proteins were analysed by SDS/polyacrylamide-gel electrophoresis followed by autoradiography. As shown in Fig. 3, autophosphorylation of the receptor β -subunit (95 kDa) was observed as early as 2 min after the addition of [γ - 32 P]ATP, and increased markedly thereafter to reach a plateau between 6 and 8 min. Quantitative analysis yielded a time of half-maximal phosphorylation of 2 min. Phosphorylation of the 110 kDa protein was first seen 4 min after the addition of [γ - 32 P]ATP, and increased subsequently to reach a maximum after a 15 min incubation. The

Fig. 3. Time course of insulin-induced phosphorylation of the insulin receptor β -subunit (95 kDa), and of the 110 kDa endogenous substrate derived from rat hepatocytes. Lectin-purified glycoproteins obtained from rat hepatocytes were exposed to insulin (1 μ M) for 90 min at 20°C, and then [γ - 32 P]ATP was added. At the times indicated, samples were taken and the reaction was stopped with boiling SDS solution. The proteins were analysed by SDS/polyacrylamide-gel electrophoresis under reducing conditions, followed by autoradiography. For key see Fig. 1 legend.

time of half-maximal phosphorylation of the endogenous substrate was 8 min.

The time necessary to reach maximal phosphorylation was chosen to study next the effect of increasing concentrations of insulin on phosphorylation of the 110 kDa protein. As shown in Fig. 4, the amount of 32 P incorporated in the 95 kDa receptor β -subunit was insulin-dependent, with an ED₅₀ for insulin of approx. 1 nM. A very similar pattern of insulin-enhanced phosphorylation was observed with the 110 kDa phosphoprotein, with an ED₅₀ of 1–2 nM.

Discussion

Promising progress in understanding the molecular basis of insulin action has been achieved by the demonstration that the insulin receptor is an insulin-sensitive kinase able to phosphorylate itself (Kasuga *et al.*, 1982a,b; Van Obberghen & Kowalski, 1982; Avruch *et al.*, 1982; Petruzelli *et al.*, 1982; Roth & Cassel, 1983; Shia & Pilch, 1983; Gazzano *et al.*, 1983; Gammeltoft *et al.*, 1984) as well as exogenous substrates (Gazzano *et al.*, 1983; Stadtmauer & Rosen, 1983; Rosen *et al.*, 1983; Zick *et al.*, 1983; Braun *et al.*, 1984). The role of the kinase activity in hormone signal transduction is not clearly established, but results have been presented indicating an altered kinase activity in patients with insulin resistance (Grunberger *et al.*, 1984; Kahn *et al.*, 1984) and in insulin-resistant mice, rendered obese by the injection of gold-thio-glucose (Y. Le Marchand-Brustel, T. Grémeaux, R. Ballotti & E. Van Obberghen, unpublished work). Furthermore, the fact that insulin-like agents, such as vanadate and trypsin (Tamura *et al.*, 1983a,b), lead to increased receptor phosphorylation favours the hypothesis that receptor phosphorylation is a crucial step in insulin action. A major challenge for the contention of a role for

Fig. 4. Dose-response of insulin effect on phosphorylation of a 110kDa endogenous protein derived from rat liver. Samples of wheat-germ-purified glycoproteins derived from rat hepatocytes were incubated for 90 min at 20°C with insulin at the concentrations (M) indicated below the gels. Thereafter, the phosphorylation reaction was initiated by adding [γ - 32 P]ATP, and was allowed to proceed for 15 min at 20°C. The reaction was stopped by addition of a boiling SDS solution, and the samples were analysed by SDS/polyacrylamide-gel electrophoresis under reducing conditions, followed by autoradiography. For key see Fig. 1 legend.

the insulin-receptor kinase in hormone action is the demonstration of cellular targets for this enzyme activity.

To approach the role of the insulin-receptor kinase in the generation of insulin effects, we have searched here for endogenous substrates. Our data provide evidence that, in lectin-purified glycoproteins prepared from rat hepatocytes or rabbit brown adipose tissue, a 110kDa phosphoprotein is found whose phosphorylation is significantly enhanced by insulin. The observation that this 110kDa protein could not be immunoprecipitated by highly specific anti-receptor antibodies indicates that this phosphoprotein is not a constituent of the insulin receptor itself. However, at the present time we cannot completely rule out that this protein is a degradation product of the receptor α -subunit (135kDa). Nevertheless, this possibility seems very remote, since under our experimental conditions we have been unable to demonstrate

32 P labelling of the α -subunit in either intact cells or cell-free systems (Van Obberghen & Kowalski, 1982; Van Obberghen *et al.*, 1983). Our data with lectin-purified glycoproteins from rabbit brown adipose tissue demonstrate that insulin-enhanced phosphorylation of this 110kDa protein is not unique for rat hepatocytes. It thus appears that this endogenous substrate is neither tissue- nor species-specific.

The insulin requirement for phosphorylation of the insulin-receptor kinase and of the 110kDa substrate is remarkably similar, and comparable with previous findings for insulin-receptor β -subunit autophosphorylation (Kasuga *et al.*, 1982a,b). Further, kinetic studies demonstrated a lag time of approx. 6 min between autophosphorylation of the insulin-receptor kinase and of the 110kDa-protein phosphorylation. This result suggests that receptor β -subunit autophosphorylation, which is thought to be necessary for activation of the receptor

kinase, leads to phosphorylation of the 110kDa protein.

The 110kDa substrate appears to be a glycoprotein composed of a single polypeptide chain, since it is purified by wheat-germ-lectin chromatography and its electrophoretic mobility is not affected after reduction of disulphide bonds. Finally, phospho-amino acid analysis of ^{32}P -labelled 110kDa phosphoprotein revealed a much higher percentage (30%) of phosphotyrosine than would have been expected in cellular proteins derived from non-neoplastic cells, which contain approx. 0.05% phosphotyrosine (Hunter & Sefton, 1980). This result, along with the fact that the only insulin-sensitive tyrosine kinase known thus far is the insulin-receptor kinase, favours the hypothesis that this 110kDa protein is an endogenous substrate for the insulin-receptor kinase. However, the putative interaction between the receptor kinase and the 110kDa protein seems to be complex, as reflected by the presence of phosphoserine and phosphothreonine in addition to phosphotyrosine. The phosphorylation of this protein might therefore result not only from the insulin-receptor tyrosine kinase, but also from other kinases modulated directly or indirectly by the receptor enzyme activity.

The identification of this 110kDa cellular protein as an endogenous substrate for the insulin-receptor kinase activity further supports the idea of a cascade of phosphorylation/dephosphorylation steps leading to insulin's biological effects. Major challenges remain the direct demonstration that this 110kDa protein is phosphorylated by the activated receptor kinase both in cell-free systems and, more importantly, in intact cells, and the elucidation of biologically function(s) of this endogenous protein substrate.

Note added in proof (received 25 February 1985)

Independently, Rees-Jones & Taylor (1985) have identified a similar endogenous substrate (M_r 120000) for the insulin receptor kinase.

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