Insulin binds to and promotes the phosphorylation of a M_r 210000 component of its receptor in detergent extracts of rat liver microsomes

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Insulin in the presence of Mn^{2+} and [γ^{32}P]ATP promoted the phosphorylation of two proteins of M_r 95000 and M_r 210000 in detergent extracts of rat liver microsomes. The M_r 210000 protein was identified as a component of the insulin receptor by immunoprecipitation. It also bound [^{125}I]insulin specifically, was phosphorylated largely on a tyrosine residue and could not be cleaved to smaller subunits under extreme reducing conditions. The M_r 210000 protein appears to be a component of a sub-population of liver membrane insulin receptors in which insulin-binding and insulin-stimulated tyrosine kinase phosphorylation site(s) reside in a single polypeptide chain.

Insulin binding *Tyrosine kinase* *Insulin receptor*
Autophosphorylation *Proreceptor* *Liver membrane*

1. INTRODUCTION

Recent investigations have established that insulin, when added to intact cells, or detergent extracts of membranes, can promote the phosphorylation of the M_r 95000 (β) subunit of its cellular receptor [1,2]. In addition, insulin added to partially purified insulin receptor preparations activates a tyrosine-specific protein kinase [3], (submitted). The insulin receptor may itself be the tyrosine kinase, in a manner analogous to the cellular receptor for epidermal growth factor (EGF) [4]. Direct evidence favoring this formulation is the demonstration of an ATP-binding site on the M_r 95000 subunit of the insulin receptor [5,6]. Since insulin appears to bind exclusively to the M_r 130000 (α) subunit of its receptor, it has been assumed that the M_r 130000 subunit is the binding subunit and the M_r 95000 subunit is the effector subunit of a disulfide-linked oligomer.

During studies in which we evaluated insulin-stimulated receptor autophosphorylation in detergent extracts of rat hepatic microsomes, we repeatedly noted insulin-stimulated phosphorylation of a minor phosphoprotein of M_r 210000 (submitted). Here, we present evidence that this M_r 210000 subunit is a component of at least a fraction of the insulin receptors in these membrane extracts, and that it binds insulin in a displaceable manner. These findings suggest that in a proportion of insulin receptors in this tissue, insulin-binding and tyrosine kinase activities may reside on a single polypeptide chain.

2. MATERIALS AND METHODS

Microsomal extracts were prepared from fed, fasted or fasted/refed male Sprague–Dawley rat (200–300 g) livers by a method described in detail elsewhere (submitted). Briefly, livers were minced, homogenized with a Teflon/glass homogenizer in an ice-cold buffer (10 mM sodium phosphate (pH 7.5), 0.25 M sucrose, 10% (v/v) glycerol, 5 mM EDTA, 10 mM benzamidine–HCl, 10 mM ϵ-aminoisocaproic acid, 0.1% (w/v) bacitracin, 10^3 KIU/ml aprotinin, 2 μM pepstatin and 2 μM

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leupeptin), and centrifuged at 12000 × *g* for 30 min. The resulting supernatant was centrifuged at 100000 × *g* for 60 min, and the microsomal pellet was resuspended in 50 mM Tris–HCl (pH 7.4), 0.25 M sucrose, at about 10 mg protein/ml, and stored at −70°C. Membranes were thawed in the presence of (final concentration) 1% (w/v) Triton X-100, 2 μM leupeptin, 2 μM pepstatin, and 10^3 KIU/ml aprotinin, extracted at 0°C for 40 min, and the supernatant of a 100000 × *g* for 60 min centrifugation was recovered. In a standard assay, the supernatants (about 10 mg protein/ml) were exposed to insulin (1 μM) or control conditions for 10 min at room temperature, followed by chilling to 0°C. This extract was added to a reaction mixture consisting of (final concentration) 7.5 mM $MnCl_2$, 50 mM Tris–HCl (pH 7.5), 16.7 mM NaF, 0.05 mM $ZnSO_4$ and 200 μM [γ-^{32}P]ATP (1500 cpm/pmol). After 5 min at 0°C, the reaction was stopped by the addition of 1% (w/v) SDS and dithiothreitol (DTT) (40 mM), or as noted in the figure legends. The samples were boiled for 5 min prior to detergent gel electrophoresis [7].

Immunoprecipitation with antiserum directed against the human insulin receptor was carried out as in [2]. To cross-link [^{125}I]insulin to its receptor,

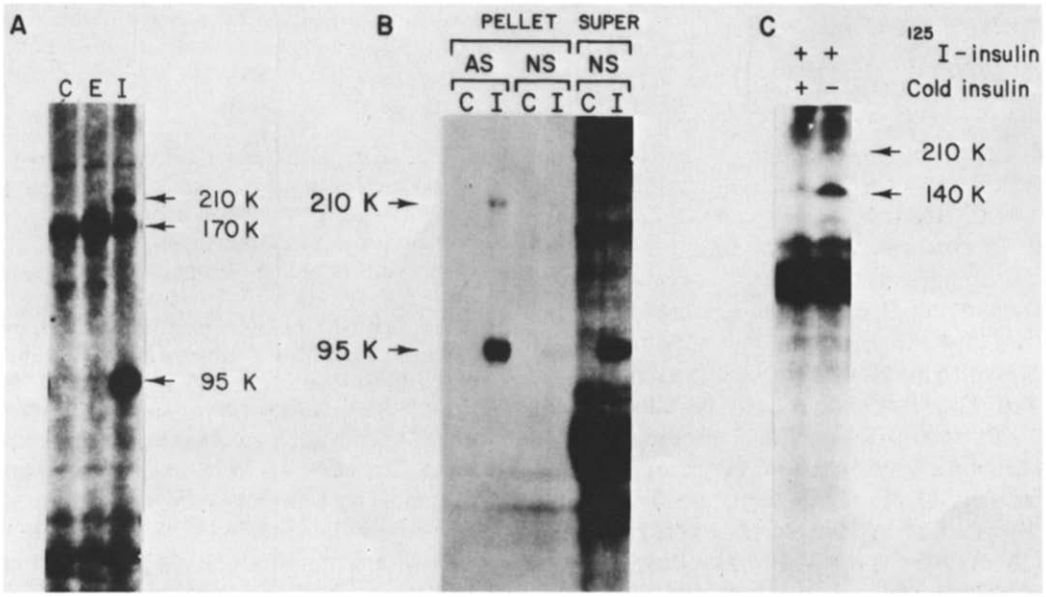

Fig. 1. Insulin-stimulated phosphorylation and immunoprecipitation of and [^{125}I]insulin binding to the M_r 210000 protein. (A) Microsomal extracts (10 mg protein/ml) from starved rat livers were exposed to control conditions (C), EGF (10^{-6} M; E) or insulin (10^{-6} M; I) for 10 min at room temperature, then phosphorylated under standard conditions as described in the text. The arrows point to the M_r 95000 β subunit of the insulin receptor (95 k), the M_r 170000 EGF-receptor (170 k), and the M_r 210000 protein (210 k). (B) Phosphorylated extracts of rat liver microsomes, exposed to control conditions (C) or insulin (I), were immunoprecipitated (PELLET) with normal human serum (NS) or anti-insulin receptor antiserum (AS) as described in the text; in addition, the supernatant of the immunoprecipitation reaction in the presence of normal serum (SUPER) is shown. The arrows point to the M_r 95000 (95 k) and the M_r 210000 (210 k) subunits of the insulin receptor. (C) [^{125}I]Insulin (10^{-10} M) was incubated with rat liver microsomal membrane extracts (10 mg/ml) for 30 min at room temperature in the presence (+) or absence (−) of excess non-radioactive insulin (10^{-6} M) (cold insulin), and then cross-linked to the insulin receptor with disuccinimidyl suberate. Proteins of M_r 210000 (210 K) and M_r 140000 (140 K) were specifically labelled with [^{125}I]insulin. The minor band of M_r ~ 115000 which appears to be specifically labelled was shown in other studies to be a proteolytic fragment of the M_r 140000 protein. A large amount of non-specific labelling was also noted on unidentified proteins of M_r of about 60000.

the membrane extract was allowed to bind [^{125}I]-insulin (10^{-10} M; spec. act. about 100 μCi/μg) for 30 min at room temperature in the presence or absence of 10^{-6} M non-radioactive insulin. Disuccinimidyl suberate was then added (final conc. 1 mM), and cross-linking was allowed to proceed for 5 min at room temperature.

The identification of ^{32}P-linked amino acid residues was carried out as in [2].

3. RESULTS

Insulin promoted the phosphorylation of two proteins in detergent extracts of rat liver microsomes, a major phosphoprotein of M_r 95 000 and a minor phosphoprotein of M_r 210 000 (fig. 1A). The latter protein was clearly distinct from the M_r 170 000 protein whose phosphorylation was stimulated by EGF in these extracts, which is presumably the EGF receptor. The insulin-stimulated increment in ^{32}P incorporated into the M_r 210 000 protein represented about 14% of total insulin-stimulated protein phosphorylation in extracts of livers from fed, starved or fasted/refed rats. Insulin-stimulated protein phosphorylation per unit protein was greatest in liver extracts prepared from 48-h starved animals, and these extracts were used in all subsequent experiments.

Antiserum directed at the human insulin receptor specifically immunoprecipitated both the insulin-stimulated M_r 210 000 and M_r 95 000 phosphoproteins, indicating that both were components of the insulin receptor (fig. 1B). In contrast, the M_r 210 000 phosphoprotein was not detected in immunoprecipitates of placental insulin receptors (not shown). [^{125}I]Insulin was specifically cross-linked to peptides of M_r 210 000 and M_r 140 000 (fig. 1C).

The time course of insulin-stimulated phosphorylation of both the M_r 210 000 and M_r 95 000 proteins was identical when [γ-^{32}P]ATP was added after pre-binding insulin (fig. 2a). This time course identity is against the possibility that the M_r 210 000 protein serves as a substrate for a protein kinase associated exclusively with the M_r 95 000 subunit. When the M_r 210 000 phosphoprotein was cut from the gel, extracted, hydrolyzed in 6 N HCl, and subjected to thin-layer electrophoresis in two dimensions, the major increment in phosphorylation promoted by insulin was on a tyrosine residue,

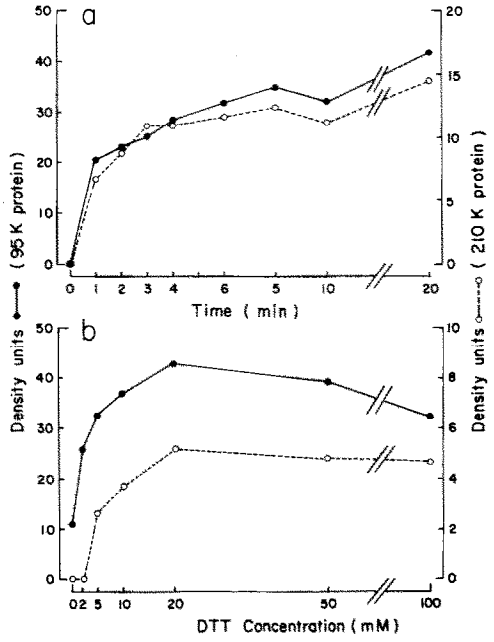

Fig. 2. Time course (a) and effect of DTT concentration (b) on the appearance of the M_r 95 000 and M_r 210 000 insulin-stimulated phosphoproteins. (a) Liver microsomal extracts were exposed to insulin (10^{-6} M) at room temperature for 10 min, followed by cooling to 0°C and addition of 7.5 mM $MnCl_2$ and [γ-^{32}P]ATP. Aliquots of the reaction mixture were removed at the specified intervals and subjected to electrophoresis. The densities of the M_r 95 000 (●—●) and M_r 210 000 (○—○) [^{32}P]proteins at each time point are shown. In (b) the effect of increasing concentrations of DTT on the appearance of the M_r 95 000 (●—●) and the M_r 210 000 (○—○) [^{32}P]proteins at their appropriate positions in the autoradiograph are shown. After phosphorylation under standard conditions after exposure to insulin, aliquots of the reaction mixture were mixed with (final concentration) 1% (w/v) SDS and the concentrations of DTT noted in the abscissa, and then boiled for 5 min. See the text for further details.

although traces of phosphoserine were noted in both control and insulin-stimulated samples (not shown).

To establish that the M_r 210 000 [^{32}P]peptide could not be further cleaved by disulfide reduction, the reaction was stopped by the addition of 1% SDS and 40 mM DTT or 1% SDS, 5% β-mercaptoethanol and 100 mM DTT [8], followed by boiling in both cases. The more stringent

disulfide-reducing conditions did not alter the relative proportions of the insulin-stimulated phosphoproteins (M_r 210 000 and M_r 95 000) in the gel (not shown). Furthermore, the relative proportions of the M_r 210 000 and M_r 95 000 phosphoproteins were unaffected over a wide range of DTT concentrations (fig. 2b), a result which would not be expected if the M_r 210 000 protein were merely an incompletely reduced heterodimer of the α- and β-subunits.

4. DISCUSSION

This study demonstrates that detergent extracts of rat liver microsomal membranes contain a M_r 210 000 protein whose tyrosine-specific phosphorylation is stimulated by the addition of insulin directly to the extract This M_r 210 000 protein is identified as a component of the insulin receptor by its ability to be specifically cross-linked to [^{125}I]insulin as well as by its immunoprecipitation by antisera directed at the insulin receptor. The M_r 210 000 protein does not appear to be a disulfide-linked heterodimer of the M_r 130 000 (α) and M_r 95 000 (β) subunits of the insulin receptor. Nevertheless, there do appear to be structural homologies between the M_r 210 000 and M_r 95 000 [^{32}P]proteins based on partial [^{32}P]peptide maps (unpublished) and based on peptide maps using other labelling procedures [8].

These findings should be compared to those in IM9 lymphocytes [8] and 3T3-L1 cells [9]. Both groups identified a M_r 210 000 protein immunoprecipitated by antisera to the insulin receptor, and suggested that it may represent a biosynthetic precursor of both the M_r 130 000 and M_r 95 000 subunit. They also provided evidence that at least a fraction of the M_r 210 000 proteins were at the cell surface, as determined by surface labelling and limited proteolysis in living cells.

We are uncertain as to whether the hepatic M_r 210 000 insulin receptor subunit observed here is a receptor precursor, or separate functional pool of mature receptor subunits. In any case, the hepatic M_r 210 000 subunit appears to be part of a subpopulation of insulin receptors in which insulin-binding and autophosphorylation activities reside on a single peptide chain, and whose role in insulin action remains to be defined.

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REFERENCES

[1] Kasuga, M., Karlsson, F.A. and Kahn, C.R. (1982) Science 215, 185–187.
[2] Avruch, J., Nemenoff, R.A., Blackshear, P.J., Pierce, M.W. and Osathanondh, R. (1982) J. Biol. Chem. 257, 15162–15166.
[3] Petruzelli, L.M., Ganguly, S., Smith, C.J., Cobb, M.H., Rubin, C.S. and Rosen, O.M. (1982) Proc. Natl. Acad. Sci. USA 79, 6792–6796.
[4] Cohen, S., Fava, R.A. and Sawyer, S.T. (1982) Proc. Natl. Acad. Sci. USA 79, 6237–6241.
[5] Roth, R.A. and Cussell, D.J. (1983) Science 219, 299–301.
[6] Van Obberghen, E., Rossi, B., Kowalski, A., Gazzano, H. and Ponzio, G. (1983) Proc. Natl. Acad. Sci. USA 80, 945–949.
[7] Blackshear, P.J., Nemenoff, R.A. and Avruch, J. (1982) Biochem. J. 204, 817–824.
[8] Kasuga, M., Hedo, J.A., Yamada, K.M. and Kahn, C.R. (1982) J. Biol. Chem. 257, 10392–10399.
[9] Deutsch, P.J., Wan, C.I., Rosen, O.M. and Rubin, C.S. (1983) Proc. Natl. Acad. Sci. USA 80, 133–136.