

# Phosphorylation–dephosphorylation of purified insulin receptor from human placenta

## Effect of insulin

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The insulin receptor of human placenta even after extensive purification is phosphorylated in the presence of [ $\gamma$ - $^{32}$ P]ATP and NaF, and is dephosphorylated again on incubation in NaF-free medium. Insulin stimulates phosphate incorporation into the  $M_r$  95 000 subunit probably by activation of the phosphorylation step. Our data suggest that the insulin receptor contains both kinase and phosphatase activities that may control the phosphorylation state of the receptor.

*Insulin receptor      Placenta      Phosphorylation      Insulin action*

### 1. INTRODUCTION

Studies on rat hepatoma cells and human lymphocytes [1], on normal rat hepatocytes [2], and also on solubilized preparations from rat liver membranes [2,3] indicate that the insulin receptor is liable to phosphorylation, and that insulin stimulates this reaction. Here, we show that the insulin receptor from human placenta undergoes also phosphorylation stimutable by insulin, and that this property is maintained even after extensive purification of the receptor protein. Moreover, our studies suggest that the purified insulin receptor contains, in addition to kinase(s), tightly bound phosphatase(s) which catalyze(s) the dephosphorylation reaction. As to the effect of insulin, evidence is presented that the hormone increases the rate of phosphorylation by activation of the kinase rather than by inhibition of the phosphatase.

### 2. MATERIALS AND METHODS

Chemicals and materials (sources in brackets): insulin, bovine, acrylamide, bisacrylamide (Serva, Heidelberg); Triton X-100, phenylmethanesulfonyl fluoride (PMSF), *N*-acetyl-D-glucosamine (Sigma,

St Louis); leupeptin (Prot. Res. Found. Osaka); adenosine 5'-[ $\gamma$ - $^{32}$ P]triphosphate([ $\gamma$ - $^{32}$ P]ATP) (NEN, Dreieichenhain); adenosine 5'-triphosphate (ATP) (Boehringer, Mannheim); silver-stain kit, hydroxyapatite (Bio-Rad, München); Sepharose 6B-Cl (Pharmacia, Uppsala); polyethylene glycol 6000 (PEG) (Roth, Karlsruhe); bovine serum albumin (BSA) (Calbiochem, Gießen).

#### 2.1. Assay of insulin binding activity (IBA)

Specific  $^{125}$ I-insulin binding was determined as in [4] using dextrane-coated charcoal for separation of free and bound ligand. In our standard assay 1 ng (=25  $\mu$ U) of  $^{125}$ I-insulin (kindly supplied by Novo, Copenhagen) was incubated in 300  $\mu$ l buffer for 45 min at 25°C at receptor concentrations within the linear range. Specific IBA is expressed as mU/mg protein.

Protein was determined by either biuret [5] or Lowry [6] procedure after precipitation with a 20-fold volume of dioxane and washing of the precipitate with 1 ml 10% trichloroacetic acid.

Standard proteins (Bio-Rad, München) for  $M_r$  determination were ( $\times 10^{-3}$ ): myosin (200);  $\beta$ -galactosidase (116); phosphorylase *b* (94); bovine serum albumin (68); ovalbumin (43).

## 2.2. Purification of the insulin receptor

Membranes were prepared from fresh human placenta and extracted with 1.5% Triton X-100 essentially as in [7]. HEPES was replaced by Tris, and leupeptin ( $10^{-6}$  M) and PMSF ( $10^{-5}$  M) was present in the buffer solutions. IBA of the Triton extracts was 0.1–0.15 mU/mg protein.

## 2.3. Gel filtration on Sepharose 6B-Cl

The  $105\,000 \times g$  supernatant of solubilized membranes ( $\sim 25$  ml corresponding to 200–300 mg protein) was passed over a Sepharose 6B-Cl column ( $4 \times 100$  cm) equilibrated with 50 mM Tris-HCl (pH 7.6) containing 0.1% Triton X-100 (T/T-buffer) at  $\sim 0.5$  ml/min. Fractions (10 ml) were collected, those with IBA were pooled and after adjusting to 10 mM  $MgCl_2$  concentrated by precipitation with PEG, final conc. 12.5% (IBA  $\sim 1$  mU/mg protein). This fraction was rechromatographed on a Sepharose 6B-Cl column ( $2 \times 170$  cm), concentrated by lyophilisation and precipitated with PEG as above (IBA  $\sim 4$  mU/mg protein).

## 2.4. Sucrose density gradient centrifugation

Preparations after gel filtration (corresponding to 1–3 mg protein) were layered onto 12.5 ml 5–20% linear sucrose gradients in T/T-buffer, and centrifuged at 41 000 rev./min at  $4^\circ C$  for 24 h (SW 41 rotor, Beckman Model L5 65 ultracentrifuge). The receptor appeared in the first three 1 ml fractions collected from bottom (IBA  $\sim 10$  mU/mg protein).

## 2.5. Chromatography on hydroxyapatite

The fraction was applied on a hydroxyapatite column ( $1 \times 4$  cm), equilibrated with T/T-buffer. Unabsorbed protein together with sucrose was washed out with T/T-buffer. The receptor was eluted with 200 ml linear gradient of 1–40 mM Na-phosphate buffer (pH 7.6) containing 0.1% Triton X-100. After lyophilisation the protein was taken up in a small volume of T/T buffer. This preparation (IBA  $\sim 20$  mU/mg protein) displayed apparent homogeneity of the  $M_r$  135 000 and 95 000 receptor subunits on SDS-PAGE (fig. 1A).

## 2.6. Polyacrylamide gel electrophoresis

Further purification was achieved by polyacrylamide slab gel electrophoresis and elution from the

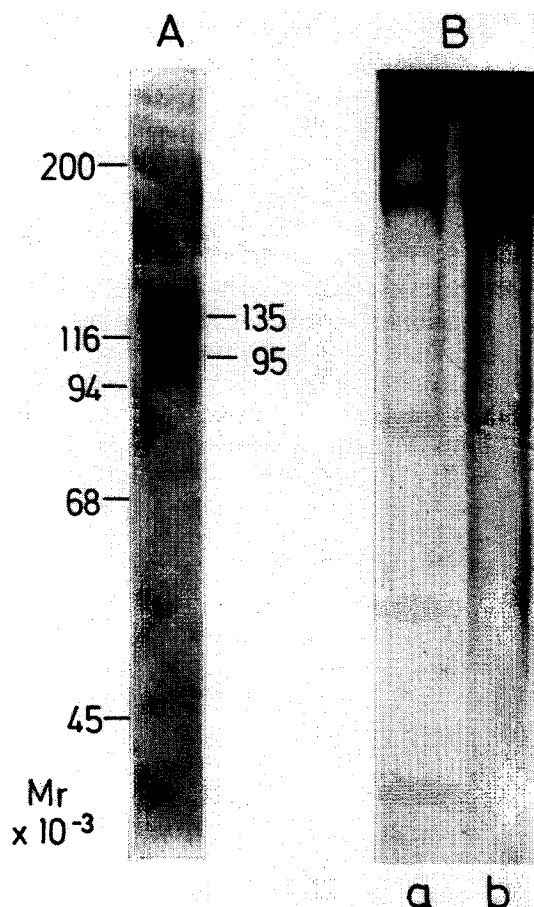


Fig. 1. Electrophoretic pattern of insulin receptor from human placenta. (A) Fraction after hydroxyapatite chromatography. SDS-PAGE ( $14 \mu g$  protein) on 7.5% slab gel according to [8]. (B) Fraction as in (A) separated on 3.75% polyacrylamide slab gel as in [8] except that SDS was replaced in the gel and buffer by Triton X-100, final conc. 0.07%. In (a)  $5 \mu g$  and (b)  $10 \mu g$  of non-reduced protein was applied. In (A) and (B) proteins were localized by silver stain [9]. For elution of protein the fraction corresponding to the undissociated receptor (see B) was cut out from unstained parallel gels and homogenized in a glass-teflon homogenizer with T/T-buffer. After centrifugation 50% of IBA with a specific activity of 40 mU/mg protein was recovered in the supernatant.

gel as in fig. 1B. IBA of these preparations was 30–40 mU/mg protein with a recovery from the gel of 50%.

### 3. RESULTS AND DISCUSSION

Placenta membranes are phosphorylated in a time-dependent manner on incubation with [ $\gamma$ - $^{32}$ P]ATP (fig. 2). The rate of phosphate incorporation is largely increased after solubilization of the membranes with Triton X-100 which also clearly enlarges the modest stimulatory effect of insulin seen with intact membranes. It appears that solubilization increases the number of phosphorylation sites of membrane proteins accessible to the action of protein kinase(s) – both insulin-dependent and -independent. The stimulation of phosphorylation of solubilized membranes was half maximal at  $10^{-9}$  M insulin reaching the maximum at  $10^{-8}$  M.

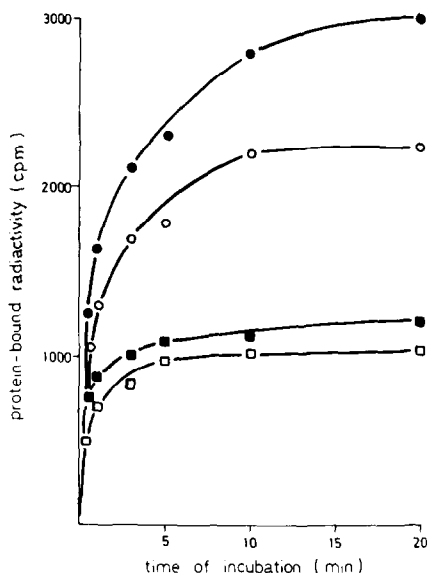


Fig. 2. Time course of  $^{32}$ P incorporation in human placental membranes before ( $\square$ ,  $\blacksquare$ ) and after ( $\circ$ ,  $\bullet$ ) solubilization with Triton X-100. Membranes corresponding to 400  $\mu$ g protein were suspended in a mixture of 50 mM Tris-HCl (pH 7.6), 12 mM  $\text{MgCl}_2$ , 1 mM  $\text{MnCl}_2$ , 100 mM NaF, 5 mM DTT, 20  $\mu$ g BSA, and when added 0.1  $\mu$ M insulin. If solubilized membranes were to be studied 1% Triton X-100 was included. After 30 min preincubation at room temperature the samples were placed on ice and the reaction was started by addition of [ $\gamma$ - $^{32}$ P]ATP ( $\sim 6$   $\mu$ Ci) to give 12–15  $\mu$ M final ATP conc. Samples were kept at 0°C, and at the times indicated 45  $\mu$ l aliquots were pipetted off for determination of protein-bound radioactivity as described in [10]: ( $\square$ ,  $\circ$ ) no insulin; ( $\blacksquare$ ,  $\bullet$ ) insulin added.

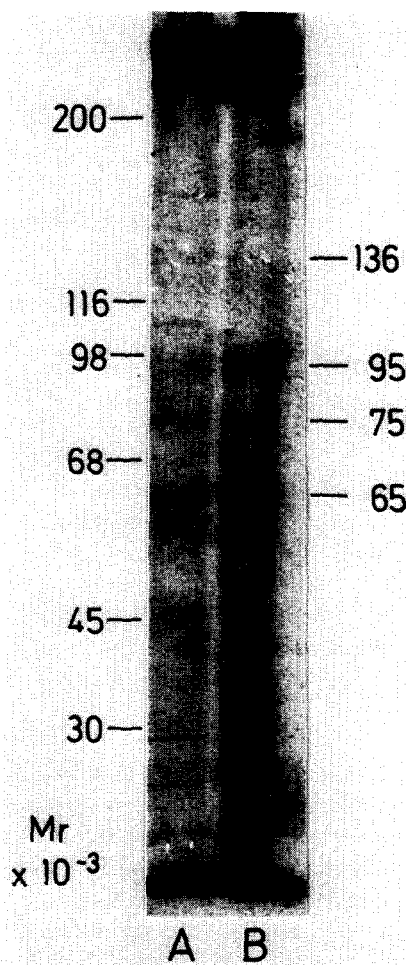


Fig. 3. Autoradiogram of human placenta membrane phosphoproteins after gel filtration and SDS-polyacrylamide gel electrophoresis. Membranes (50 mg protein) were preincubated in the Triton X-100 containing medium described in fig. 1 in the absence (A) or presence (B) of insulin ( $10^{-7}$  M). [ $\gamma$ - $^{32}$ P]ATP (250  $\mu$ Ci, final conc. 15  $\mu$ M) was added and incubation (at 0°C) continued for 10 min. Reaction was stopped by addition of EDTA, final conc. 25 mM. The receptor was purified on Sepharose 6B-Cl as described in the method section except that the first step was also done on the  $2 \times 170$  cm column. The pooled peak fractions (200  $\mu$ g protein) were mixed with rabbit serum containing insulin receptor antibodies (preparation to be described elsewhere), and precipitated with a second antibody (sheep anti-rabbit IgG, Behring, Marburg). The precipitate was resuspended and washed with trichloroacetic acid and further processed for SDS-PAGE as in fig. 1 and autoradiography on Kodak X-Omat films as in [11].

$K_M$  app. for ATP was  $3 \mu\text{M}$  (not shown). During chromatography of the phosphorylated membrane proteins on Sepharose 6B-Cl the peak of the radioactivity coincided with the peak of IBA ( $M_r$  350000), the specific radioactivity (cpm/mg protein) being about twice as high after pre-treatment of membranes with insulin. Further evidence that the receptor protein is involved was obtained on examination by SDS-polyacrylamide gel electrophoresis and autoradiography (fig. 3). In agreement with [2] it is shown that it is the  $M_r$  95000-subunit of the insulin receptor whose phosphorylation is stimulated by insulin under these conditions.

Insulin-stimulated phosphorylation is demonstrable with receptor preparations carried through all purification steps up to the highest degree of purity. This is documented by the experiments shown in fig. 4 which were performed with apparently homogenous receptor preparations eluted from polyacrylamide gel after electrophoresis (see fig. 1B). Moreover, these experiments show that

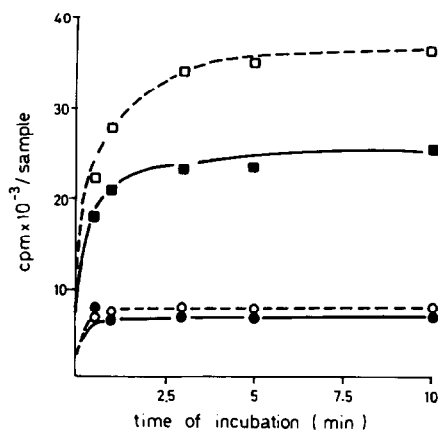


Fig. 4. Effect of insulin on phosphorylation of highly purified insulin receptor in the presence (□, ■) and absence (○, ●) of NaF:  $9.5 \mu\text{g}$  electrophoretically purified insulin receptor (see section 2.6 and fig. 1B) dissolved in  $400 \mu\text{l}$  solution composed of  $50 \text{ mM}$  Tris-HCl (pH 7.6),  $12 \text{ mM}$   $\text{MgCl}_2$ ,  $1 \text{ mM}$   $\text{MnCl}_2$ ,  $5 \text{ mM}$  DTT,  $18 \mu\text{g}$  BSA,  $0.1\%$  Triton X-100, and where added  $100 \text{ mM}$  NaF. After preincubation (45 min,  $25^\circ\text{C}$ ) without (■, ●) and with insulin ( $10^{-7} \text{ M}$ ) (□, ○) the samples were placed on ice and after addition of  $20 \mu\text{Ci}$   $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ , final conc.  $15 \mu\text{M}$ , phosphorylation was studied as in fig. 1.

phosphorylation is largely decreased at omission of NaF from the incubation suggesting that there is also a phosphatase(s) present. The failure of insulin to increase phosphate incorporation in the absence of NaF (fig. 4) would then suggest that insulin enhances receptor phosphorylation by stimulation of kinase(s) rather than by inhibition of phosphatase(s).

Fig. 5 illustrates that the phosphorylated insulin receptor is dephosphorylated again upon incubation in NaF-free medium. This lends further support to the assumption that besides a kinase(s) there is also a phosphatase(s) tightly associated with the receptor catalyzing a phosphorylation-dephosphorylation cycle resembling the operation of some interconvertible enzyme systems. The failure

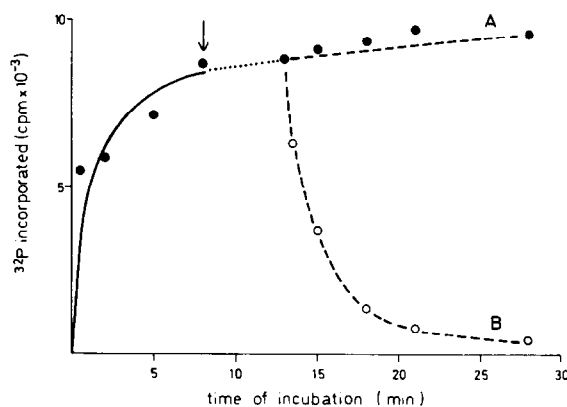


Fig. 5. Phosphorylation-dephosphorylation of highly purified insulin receptor from human placenta. Receptor  $400 \mu\text{l}$  in T/T-buffer after hydroxyapatite chromatography ( $28 \text{ mU/mg}$  protein) (see section 2.5 and fig. 1A) corresponding to  $54 \mu\text{g}$  protein was mixed with  $155 \mu\text{l}$   $50 \text{ mM}$  Tris-HCl (pH 7.6),  $80 \mu\text{l}$   $1 \text{ M}$  NaF,  $20 \mu\text{l}$   $0.1 \text{ M}$   $\text{MnCl}_2$ ,  $20 \mu\text{l}$   $0.5 \text{ M}$   $\text{MgCl}_2$ ,  $40 \mu\text{l}$   $0.1 \text{ M}$  DTT,  $40 \mu\text{l}$   $0.5\%$  BSA,  $45 \mu\text{l}$  ( $5 \mu\text{Ci}$ )  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$  (final conc.  $25 \mu\text{M}$ ). After 8 min incubation at  $0^\circ\text{C}$  (●—●) two samples of  $300 \mu\text{l}$  each were taken and rapidly mixed with  $200 \mu\text{l}$   $50\%$  PEG solution for receptor precipitation (→). After centrifugation the precipitates were washed twice with  $250 \mu\text{l}$   $20\%$  PEG in the above phosphorylation mixture except  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ : (A) was resuspended in  $300 \mu\text{l}$  of the washing medium; in (B) NaF was omitted and replaced by medium. A (●—●) and B (○---○) were then further incubated at  $0^\circ\text{C}$ . Protein bound radioactivity was determined [10] using  $45 \mu\text{l}$  samples drawn at the times indicated on the abscissa.

of detecting these regulatory enzymes as distinct proteins (e.g., on SDS-PAGE) may be explained on presuming that either there are only few present or that they represent catalytically active domains of the receptor protein itself.

Here we provide evidence that the human insulin receptor represents an interconvertible protein whose phosphorylation state depends on the relative activities of (a) kinase(s) and (a) phosphatase(s) that we have shown to strongly co-purify with the receptor protein. Our studies suggest that the increase in receptor phosphorylation by insulin is achieved through stimulation of kinase activity. The physiological significance of this interesting system, self-regulated by insulin, remains to be established.

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