An M_r 180000 protein is an endogenous substrate for the insulin-receptor-associated tyrosine kinase in human placenta

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The β-subunit of the insulin receptor contains a tyrosine-specific protein kinase. Insulin binding activates this kinase and causes phosphorylation of the β-subunit of the insulin receptor. It is believed that phosphorylation of other proteins might transmit the insulin signal from the receptor to the cell. In the present study we used a polyclonal anti-phosphotyrosine antibody to detect other proteins that become tyrosine phosphorylated upon insulin stimulation. Glycoproteins from human placenta membranes were enriched by wheat germ agglutinin chromatography and phosphorylation was studied with [γ-^{32}P]ATP and insulin in vitro. Phosphorylated proteins were immunoprecipitated by antibodies against the insulin receptor and by serum containing the anti-phosphotyrosine antibody. Beside the insulin-stimulated phosphorylation of the 95 kDa β-subunit of the insulin receptor, an insulin-stimulated phosphorylation of a 180 kDa protein was found. The phosphorylation of both proteins occurred only on tyrosine residues. Insulin increased ^{32}P incorporation into the 180 kDa band 2.7-fold (S.E.M. ±0.3, $n = 5$). The 180 kDa protein was not precipitated by antibodies against the insulin receptor. H.p.l.c. chromatograms of tryptic fragments of the phosphorylated 180 kDa protein and of the β-subunit of the insulin receptor revealed different patterns for both proteins. Insulin-stimulated phosphorylation of the 180 kDa protein was also detectable in unfractionated detergent-solubilized membranes. The phosphorylation of the 180 kDa protein was stimulated by insulin with the same dose–response curve as the phosphorylation of the β-subunit, suggesting that this protein might be another endogenous substrate of the insulin receptor kinase.

INTRODUCTION

Tyrosine-specific protein kinase activity, which was originally found to be associated with the transforming proteins of a number of different tumour viruses [1–3] has been shown to be characteristic of normal cellular homologues of the transforming proteins [4] and of the membrane receptors for a variety of growth factors [5,6] as well as for the insulin receptor [7,8].

Insulin binding to the α-subunit of the insulin receptor stimulates phosphorylation of the β-subunit of the receptor in intact cells [7–9] and cell-free systems [9–15] at tyrosine residues. It is clear now that this is due to the stimulation of a tyrosine-specific protein kinase which is part of the β-subunit of the insulin receptor [13,14]. This kinase undergoes insulin-stimulated autophosphorylation at tyrosine residues and it appears that this activates the enzyme in vitro against other substrates [15]. It is believed that phosphorylation of other proteins in the intact cell transmits the insulin signal. Despite much effort to identify tyrosine-phosphorylated substrates of the insulin receptor kinase in the intact cell, the signal-transmitting protein has not yet been found. Using anti-phosphotyrosine antibodies, White et al. [16] were recently able to detect a tyrosine-phosphorylated 185 kDa protein in a hepatoma cell line. Using the same approach we found a tyrosine-phosphorylated 46 kDa protein in rat fat cells [17]. Here we show now that a 180 kDa protein becomes phosphorylated at tyrosine residues in an insulin-stimulated way in human placenta extracts.

MATERIALS AND METHODS

Materials

Pig insulin was purchased from Novo Industrie (Bagsvaerd, Denmark), [γ-^{32}P]ATP (2900 Ci/mmol) was from NEN (Dreieich, Germany), aprotinin, phenylmethanesulphonyl fluoride, leupeptin, phosphoamino acids and Triton X-100 were from Sigma (Munich, Germany). All other reagents for SDS/PAGE were from Bio-Rad; all other reagents were of the best grade commercially available.

Methods

The solubilization and purification of insulin receptor from human placenta membranes, the assay of insulin-binding activity, protein determination and SDS/PAGE were performed as described [10,18,19].

Phosphorylation assay of placenta membrane proteins

Solubilized human placenta membranes, in buffer containing 50 mM-Tris/HCl, 0.1% Triton X-100, 0.1 mM-phenylmethanesulphonyl fluoride, 1 mM-sodium vanadate, 12 mM-MgCl$_2$ and 5 mM-MnCl$_2$, were preincubated

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Abbreviations used: WGA, wheat germ agglutinin; PAGE, polyacrylamide-gel electrophoresis; EGF, epidermal growth factor; PDGF, platelet-derived growth factor.

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without or with insulin (5×10^{-7} M) at 20 °C for 30 min. The phosphorylation reaction was then initiated by the addition of 200 μM-[γ-^{32}P]ATP and terminated after 15 min at 23 °C by the addition of 20 mM-sodium pyrophosphate, 1 mM-dithiothreitol, 20 mM-EDTA, 1 mM-sodium vanadate and 1 mM-ATP. Specific immunoprecipitates of the phosphoproteins were obtained with anti-phosphotyrosine antibodies, prepared according to the method of Pang et al. [20] or patient serum containing anti-(insulin receptor) antibodies (B_2). After incubation of the antibodies with the phosphoproteins at 4 °C for 14 h, the antibodies were immobilized on Pansorbin (Calbiochem) and the precipitates were washed three times with a solution containing 50 mM-Tris/HCl, 1% Triton X-100, 0.1% SDS and 150 mM-NaCl. Proteins were eluted from the Pansorbin with 2% SDS/25 mM dithiothreitol/3% glycerol, boiled for 15 min and separated by SDS/PAGE (5–10% acrylamide gradient). The phosphoproteins were identified by autoradiography of the stained and dried gel with Kodak X-Omat film.

Phosphorylation assay of WGA-purified placenta membrane proteins

Placenta membrane proteins solubilized with Triton X-100 (1% v/v) as described above were passed over a WGA column. The glycoprotein fraction was then eluted with N-acetylglucosamine (0.3 M). Aliquots of 5 μg were used in each assay. The phosphorylation assay was performed as described above, with the only difference that [γ-^{32}P]ATP was used at a concentration of 50 μM. Immunoprecipitation and SDS/PAGE were performed as described above.

Identification of phosphoamino acids

The protein bands of 95 kDa corresponding to the β-subunit of the insulin receptor and of 180 kDa were identified by autoradiography as indicated above. The corresponding bands in the dried gel were excised and washed with dioxan, followed by methanol and 10% (v/v) methanol. The gel fragments were placed into 200–500 μl of 6 M-HCl and hydrolysed for 2 h at 110 °C. The samples were then diluted with 2 ml of water, freeze-dried and redissolved in 30–50 μl of water. Electrophoresis was performed on Whatman 3 MM paper at pH 3.5, with pyridine/acetic acid/water (1:10:189, by vol.) for 90 min at 1 kV. Phosphoamino acid standards were localized with ninhydrin and ^{32}P-labelled amino acids by autoradiography.

Tryptic peptide mapping by h.p.l.c.

Fixed and dried polyacrylamide-gel fragments containing the 95 kDa (pp 95) β-subunit of the receptor and the 180 kDa protein, located by autoradiography after phosphorylation as described, were washed for 12 h at 37 °C with 20 ml of 10% (v/v) methanol. The gel was dried at 70 °C for 60 min and rehydrated in 0.5 ml of 200 mM-NH$_4$HCO$_3$ containing 100 μg of trypsin (type XIII from Sigma). The mixture was incubated for 20 h at 37 °C, followed by an additional 4 h incubation with 50 μg of trypsin. The gel fragments were removed and the supernatant was clarified by centrifugation (10000 g for 2 min). The supernatant was freeze-dried and the residue was dissolved in 25–50 μl of 0.1% trifluoroacetic acid and again clarified by centrifugation and filtration. The phosphopeptides were separated in a LKB h.p.l.c. system
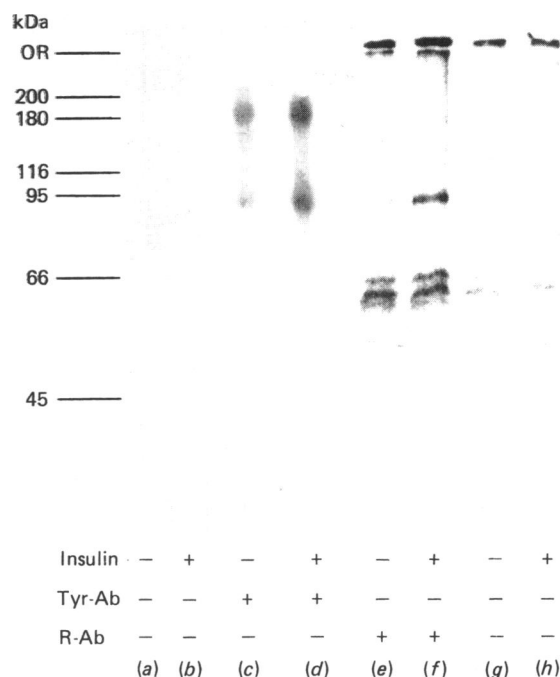

Fig. 1. Immunoprecipitation of the insulin-stimulated phosphotyrosine proteins in human placental membrane

Triton X-100 (0.1%) extracts of placental membranes (70 μg of protein) were incubated at 23 °C for 30 min in the absence or presence of 5×10^{-7} M-insulin. The reaction was initiated by the addition of 200 μM-[γ-^{32}P]ATP, and phosphorylation was terminated by addition of stop solution (as described in the Materials and methods section). Immunoprecipitates with anti-phosphotyrosine (Tyr-Ab) (lanes c and d), normal rabbit sera (lanes a and b), sera from a patient with antibodies against the insulin receptor (R-Ab) (lanes e and f) or normal human sera (lanes g and h) are shown. Bands were cut off the gel and counted for radioactivity. Radioactivity incorporated into the 180 kDa and 95 kDa bands after subtracting the background were: 180 kDa (c), 900 c.p.m., (d), 1800 c.p.m.; 95 kDa, (c), 400 c.p.m., (d), 1800 c.p.m., (e), 300 c.p.m., (f), 1500 c.p.m. This experiment was repeated three times with very good reproducibility. Abbreviation: OR, origin.

equipped with an Ultropac TSK ODS-120 T column (5 μm). Phosphopeptides applied to the column were eluted at a flow of 0.8 ml/min with water modified with acetonitrile, both containing 0.05% trifluoroacetic acid. The radioactivity of the peptides eluted from the reversed-phase column in fractions of 0.10–0.11 ml was measured in 3 ml of scintillation mixture.

RESULTS

The autoradiogram in Fig. 1 shows phosphorylated proteins from human placenta. The proteins of human placental membranes were solubilized with Triton X-100, preincubated with or without insulin (5×10^{-7} M) and subsequently phosphorylated with [γ-^{32}P]ATP. The reaction was stopped as described in the Materials and methods section and the phosphoproteins were then immunoprecipitated either with antibodies against the insulin receptor (lanes e and f) or with the polyclonal anti-phosphotyrosine antibody (lanes c and d). In the
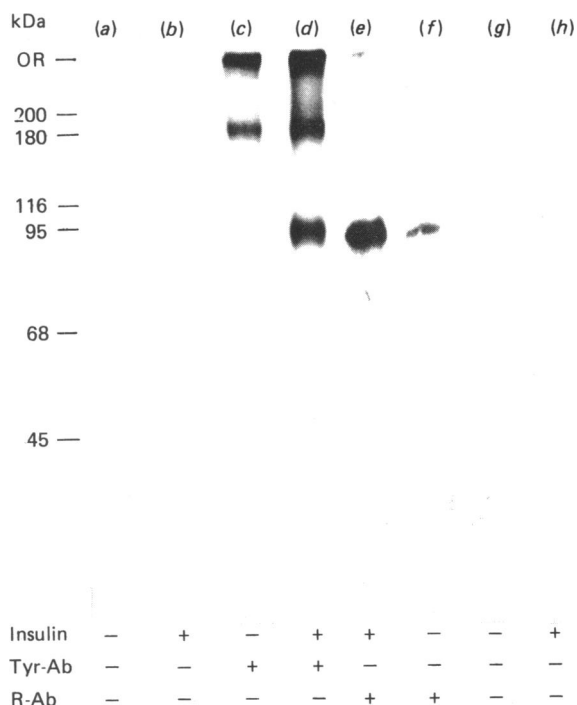
Fig. 2. Immunoprecipitation of the insulin receptor and phospho-tyrosine-containing proteins from a WGA-purified extract of human placenta membrane proteins

WGA-purified extracts (10–15 μg of protein) were pre-incubated without or with insulin (5 × 10^{-7} M) at 20 °C for 30 min. The phosphorylation reaction was then initiated by the addition of 50 μM-[γ-^{32}P]ATP and terminated after 10 min at 23 °C by the addition of stop solution. The lanes show immunoprecipitates with normal rabbit sera (lanes a and b), anti-phosphotyrosine antibody (lanes c and d), normal human sera (lanes g and h) or anti-(insulin receptor) antibody (lanes e and f). For abbreviations see Fig. 1.

Fig. 3. Dose–response curve of the insulin effect on the phosphorylation of the 95 kDa receptor band ($\bigcirc$) and the 180 kDa band ($\bullet$)

The phosphorylation was performed with WGA-purified extract of human placenta membrane proteins as described in the Materials and methods section and the legend of Fig. 2. Phosphoproteins were then immunoprecipitated with anti-phosphotyrosine antibody, separated by SDS/PAGE and identified by autoradiography. The bands corresponding to the phosphorylated β-subunit (95 kDa) and to the 180 kDa protein were excised from the gel and their radioactivity was measured. The mean values found in five separate experiments were 3950 c.p.m. (no insulin) and 10682 c.p.m. (10^{-6} M-insulin) for the 180 kDa band, and 2400 c.p.m. (no insulin) and 11000 c.p.m. (10^{-6} M-insulin) for the 95 kDa band, after subtracting the background.

immunoprecipitates of the insulin receptor antibody an insulin-stimulated phosphoprotein is detected at 95 kDa, which was earlier identified as the β-subunit of the insulin receptor of human placenta [10], as well as a nonstimulated band at around 60 kDa of unknown identity which is inconsistently found in the immunoprecipitates.

The immunoprecipitates of the phosphotyrosine antibody show the same 95 kDa band. In addition, these immunoprecipitates show a 180 kDa phosphoprotein. The 180 kDa band was cut from the gel and the ^{32}P incorporation was measured by scintillation counting. We found that insulin stimulated 2-fold the ^{32}P incorporation in this band. Due to a high background activity of other phosphoproteins the insulin effect on phosphorylation of this band was not detectable without immunoprecipitation. An insulin-stimulated phosphorylation of this protein can also be shown if the membrane proteins were first partially purified by WGA affinity chromatography. Fig. 2 shows the autoradiogram of the phosphorylation of partially purified membrane proteins which were first passed over a WGA affinity column. The proteins were phosphorylated for 10 min with [γ-^{32}P]ATP and then immunoprecipitated either with anti-(insulin receptor) antibody (lanes e and f) or with the
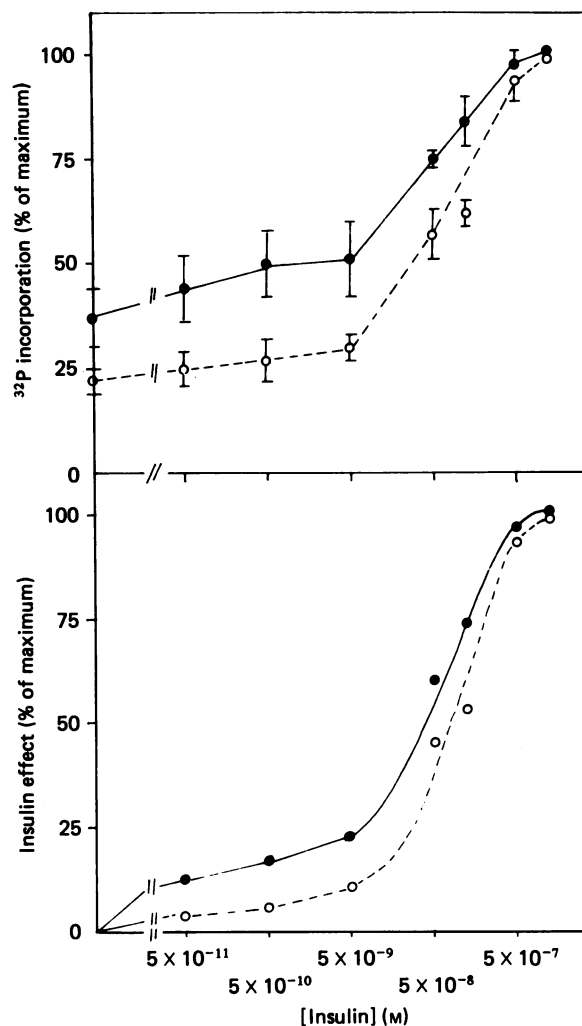
anti-phosphotyrosine antibody (lanes c and d). In the immunoprecipitates with the insulin receptor antibody again only a 95 kDa band is detectable. In contrast, the immunoprecipitates with the anti-phosphotyrosine antibody contain, in addition to the 95 kDa protein, also a phosphorylated 180 kDa band.

The 180 kDa band and the 95 kDa band were cut from the gel and the ^{32}P incorporation was measured with scintillation counting. We found that insulin stimulated ^{32}P incorporation into the 180 kDa band by a factor of
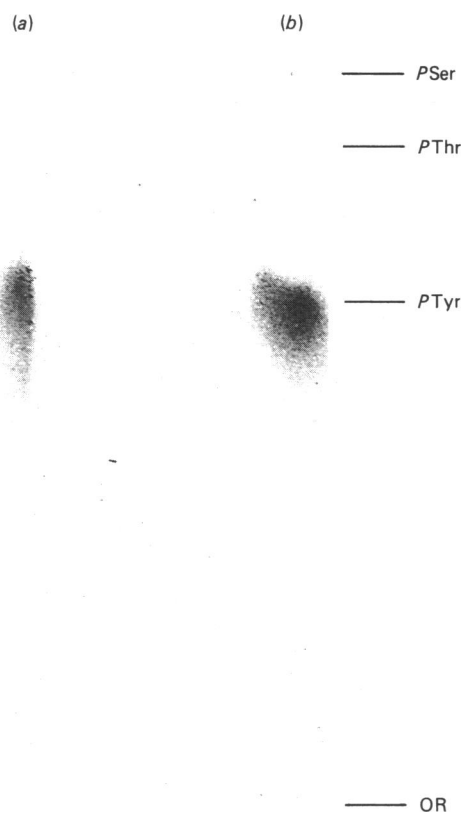
Fig. 4. Identification of phosphoamino acids

The protein bands corresponding to the 180 kDa and 95 kDa proteins from the dried gel were excised and washed with dioxan followed by methanol and 10% (v/v) methanol respectively. The gel fragments were placed into 200–500 μl of 1 M-HCl and hydrolysed for 2 h at 110 °C. Electrophoresis was performed on Whatman 3 MM paper at pH 3.5 with pyridine/acetic acid/water (1:10:189, by vol.) for 90 min at 1 kV. Phosphoamino acid standards were localized with ninhydrin, and ^{32}P-labelled amino acids by autoradiography. Lane (a) shows the hydrolysate of the 180 kDa protein (2900 c.p.m. were applied), and lane (b) the 95 kDa protein (4000 c.p.m.). Abbreviations: OR, origin; PSer, phosphoserine; PThr, phosphothreonine; PTyr, phosphotyrosine.

Fig. 5. H.p.l.c. tryptic peptide maps of the 180 kDa protein and the 95 kDa protein (β-subunit)

The gel fragments containing the two proteins from Fig. 2, located by autoradiography after phosphorylation, were incubated with trypsin for 24 h and further processed as described in the Materials and methods section. The phosphopeptides were separated by h.p.l.c. The acetonitrile gradient used was 0–40%. Phosphopeptides from the 180 kDa protein are shown as ○, and from the 95 kDa protein (β-subunit) as ●. The Figure represents the results of an experiment that was reproduced four times with different insulin receptor preparations.

2.7±0.3 ($n = 5$), compared with a 4.6-fold (± 0.5) stimulation for the 95 kDa band. Insulin stimulates the ^{32}P incorporation in both bands in a dose-dependent way. Fig. 3 shows a comparison of the dose–response curves of the insulin effect on phosphorylation of the 95 kDa protein and the 180 kDa protein. The dose–response curves of the insulin effect on phosphorylation of both proteins are similar. Half-maximal stimulation occurs at 5×10^{-8} M and the maximal insulin effect is reached at 5×10^{-7} M. In order to analyse the phosphoamino acid composition of the two phosphorylated proteins we excised the respective bands from the gel and determined the phosphorylated amino acids as described in the Materials and methods section. Fig. 4 shows an autoradiogram of a paper electrophoresis of the phosphorylated amino acids. It appears that the only phosphorylated amino acid in both proteins is tyrosine. In order to gain further information about the nature of the 180 kDa protein, we prepared tryptic fragments of
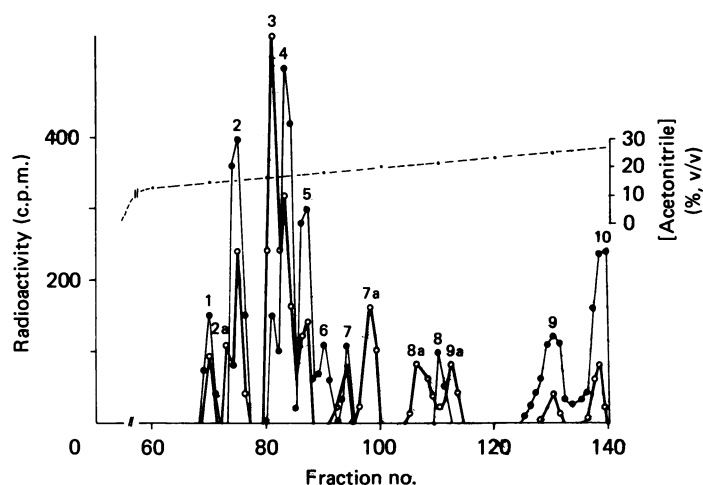
these proteins and separated them by reverse-phase h.p.l.c. Fig. 5 shows the h.p.l.c. patterns of the 180 kDa protein in comparison with the h.p.l.c. profile of the β-subunit of the insulin receptor. Even though there are overlapping regions of the elution patterns, it appears unlikely that the 95 kDa protein is derived from the 180 kDa protein.

DISCUSSION

Insulin stimulates the phosphorylation of a 180 kDa protein in a wheat germ extract from human placenta membranes as well as in a crude plasma membrane fraction. Two major questions arise: is the enzyme phosphorylating this protein the intrinsic tyrosine kinase of the insulin receptor, and what is the identity of this 180 kDa protein?

The tyrosine-specific protein kinase of the insulin receptor has been shown to phosphorylate in vitro a number of exogenous substrates [10,12,13–15]. So far only three endogenous substrates have been described [16,17,21,22]: a 185 kDa protein in intact hepatoma cells [16], a 120 kDa protein in an extract of hepatocytes purified by WGA [21,22] and a 46 kDa protein in intact rat adipocytes [17]. The 185 kDa protein found in hepatoma cells does not bind to WGA [16], suggesting that it is not identical with the protein we have found here in placenta membranes. On the other hand, this does not fully exclude an identity of both proteins, as the possibility of a difference in glycosylation between the two tissues has to be considered. The nature of the 180 kDa protein is unclear. Possible candidates in this molecular size range are the insulin receptor precursor [23], the EGF receptor [5] as well as the PDGF receptor

[6]. The observation that the 180 kDa protein is not immunoprecipitated by the insulin receptor antibody makes it unlikely that it is the insulin receptor precursor. Furthermore, analysis of the h.p.l.c. profile of the 180 kDa protein supports the same conclusion. Even though the profiles of the 180 kDa and the 95 kDa proteins contain overlapping regions, it is possible that this is due to structural similarities of the phosphorylated sites from phosphotyrosine-containing proteins. It should also be noted that peaks 4 and 5 from the receptor β-subunit, which undergo up to 10-fold stimulation by insulin in human placenta (J. M. Carrascosa & B. Obermaier, unpublished work), are not the main phosphorylated peptides of the 180 kDa protein. Other known tyrosine-phosphorylated proteins in this molecular size range are the PDGF receptor and the EGF receptor. However, neither PDGF nor EGF stimulated the phosphorylation of the 180 kDa protein in our system (results not shown). Thus, the identity of the protein is completely unclear at present.

For the protein found here, as well as for the other proteins mentioned above, no direct evidence exists that the enzyme phosphorylating them is indeed the insulin receptor kinase. However, the phosphorylation of these proteins is stimulated by insulin and occurs exclusively on tyrosine residues, and the only tyrosine-specific insulin-stimulated kinase known so far is the intrinsic insulin receptor kinase. Furthermore, some evidence supporting this idea is derived from the observation that the dose-response curves of the insulin effect on receptor phosphorylation and phosphorylation of the 180 kDa protein are identical.

In summary, our data show that there is an insulin-stimulated tyrosine-phosphorylated protein other than the insulin receptor in human placental membranes. Its identity and its possible physiological role have to be determined in further studies.

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