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Tyrosine phosphorylation of the receptor for insulin-like growth factor II is inhibited in plasma membranes from insulin-treated rat adipocytes

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INTRODUCTION

One of the major effects of insulin on its target cells is a rapid increase in the expression of several membrane proteins at the cell surface. This effect involves a redistribution of these proteins from an intracellular membrane compartment (low-density microsomal fraction) to the cell surface membrane. Three of the proteins that are known to be affected in this manner in response to insulin are the glucose transporter [1–3], the IGF-II receptor [4,5] and the transferrin receptor [6].

Isotopic labelling experiments using [32P]phosphate have shown that IGF-II receptors in plasma membranes are phosphorylated in vivo on serine and threonine residues, to a higher stoichiometry than the receptors found in low-density microsomal membranes. The redistribution of IGF-II receptors that occurs on exposure of intact cells to insulin correlates with a rapid decrease in the phosphoserine and phosphothreonine contents of the receptors which reside on the plasma membrane [7,8]. The phosphorylation of the IGF-II receptor in isolated rat adipocyte plasma membranes has also been studied. It has been found that, on incubation of the membranes with [γ-32P]ATP, the receptors become phosphorylated on tyrosine residues. This phosphorylation is catalysed by a potent tyrosine kinase activity endogenous to the adipocyte plasma membrane [9].

In the present paper we report that insulin treatment of intact adipocytes results in a pronounced and rapid inhibition of tyrosine phosphorylation of IGF-II receptors in subsequently isolated plasma membranes.

MATERIALS AND METHODS

Preparation of cells and plasma membranes

Plasma membranes were prepared from isolated adipocytes obtained from 75–100 g Sprague–Dawley rat (Taconic Farms) epididymal fat-pads as previously described [10]. Where indicated, cells were exposed to 0.1 μM-insulin for 10 min at 37 °C. The cell suspensions were then centrifuged (500 g for 2 min), and the buffer was aspirated. Cells were homogenized in 8 vol. of a buffer containing 0.25 M sucrose, 10 mM-Tris/HCl, 5 mM-EDTA and 100 μM-phenylmethanesulphonyl fluoride, pH 7.4 at 5 °C. Plasma membranes were separated as described previously [4,11]. Protein concentrations were determined by the Bradford [12] procedure (Bio-Rad).

Phosphorylation of the IGF-II receptor

(a) Intact plasma membranes (final concn. 2 mg of protein/ml) were resuspended in a buffer (phosphorylation buffer) containing 25 mM-Hepes, 80 mM-β-glycerophosphate, 10 mM-MgCl2, 2 mM-MnCl2, 100 μM-phenylmethanesulphonyl fluoride and 10 mM-ammonium molybdate (Sigma), pH 7.4 at 5 °C. Portions (50 μl) of the membrane suspension were used for each sample. Phosphorylation reactions were started by the addition of 5 μl of a 1 mM stock of [γ-32P]ATP (Amersham) to give a final concentration of 100 μM (10 μCi/nmol), and continued for 10 min at 5 °C. Reactions were terminated by addition of 500 μl of solubilizing buffer, containing 25 mM-Hepes, 1.5% Triton X-100, 1% sodium deoxy-

Abbreviation used: IGF, insulin-like growth factor.
cholate, 0.1% SDS, 1% bovine serum albumin, 0.5 M-
NaCl, 50 mM-NaF, 100 μM-Na₃VO₃, 50 mM-Na₂HPO₄, 5 mM-EDTA — and 100 μM-phenylmethanesulphonyl
fluoride, pH 7.4 at 5 °C.

(6) Triton X-100-solubilized plasma membranes were
prepared by vigorously vortex-mixing intact membranes
(2 mg/ml) in the phosphorylation buffer described
above, containing 1% Triton X-100 (Bio-Rad), for
30 min at 55 °C. The phosphorylation reactions were
started by the addition of [γ-32P]ATP as described above.
After incubation for the times indicated in each Figure
legend, the reactions were stopped by the addition of
500 μl of solubilizing buffer.

(c) The experiments shown in Figs. 4 and 6 illustrate
the phosphorylation of the IGF-II receptor present in
the Triton X-100-insoluble membrane fractions. For these
experiments, plasma membranes were resuspended
(2 mg/ml) in phosphorylation buffer containing 1%
Triton X-100. After incubation for 30 min at 5 °C, the
suspensions were centrifuged at 5000 g for 10 min.
Supernatants were removed, and the Triton X-100-
soluble pellets were gently resuspended in 50 μl of
phosphorylation buffer at 5 °C, with a 27-gauge needle
and syringe. Phosphorylation reactions were performed
as described above.

**Immunoprecipitation and SDS/polyacrylamide-gel
electrophoresis**

After the addition of solubilizing buffer, approx. 10 μg
of rabbit polyclonal anti-(IGF-II receptor) immunoglo-
bulin coupled to 20 μl of Affi-Gel 10 (Bio-Rad) was
added to each sample. Immunoadsorptions were
performed overnight at 4 °C on an end-over-end mixer.
The resin was then washed twice with solubilizing buffer and
once with 25 mM-Hepes/0.15% Triton X-100/0.1%
SDS, pH 7.4. The samples were then boiled for 2 min
in 50 mM-Tris/HCl/3% SDS/0.005% Bromophenol Blue/
20% (v/v) glycerol, pH 6.8, and separated on a 6%-
Gels were then stained with Coomassie Brilliant Blue,
dried and exposed to Kodak X-OMAT film at −70 °C.

**Phosphoamino acid analysis**

Gel slices corresponding to autoradiography bands
were excised and subjected to tryptic digestion in 0.1 M-
N-ethylmorpholine acetate, pH 8.3, for 24 h at 37 °C.
The tryptic peptides were freeze-dried to dryness,
resuspended and hydrolysed in 6 M-HCl for 75 min at
110 °C. The hydrolysates were dried, resuspended in a
solution containing phosphoserine, phosphothreonine
and phosphotyrosine (1 mg/ml each), and spotted on
thin-layer cellulose plates (Brinkman Instruments).
Electrophoresis was then performed for 120 min at
1000 V with a running buffer of acetic acid/pyridine/
water (10:1:189, by vol.). Plates were then dried, spots
detected by ninhydrin staining, and the plates auto-
radiographed at −70 °C.

**RESULTS**

Fig. 1 shows the effect of brief insulin treatment of
intact adipocytes on the subsequent phosphorylation of
IGF-II receptors in plasma membranes prepared from
these cells. Triton X-100-solubilized plasma membranes
were employed because in previous experiments it was
found that the detergent enhanced the amount of

**[32P]phosphate incorporated into the IGF-II receptor [9].**
After addition of 100 μM-[γ-32P]ATP to the membranes,
IGF-II-receptor phosphorylation increased linearly over
a 10 min period at 5 °C. The rate at which 32P was
incorporated into IGF-II receptors was greatly decreased
in membranes derived from insulin-treated cells com-
pared with those derived from control cells.

The identities of the phosphorylated residues on the
IGF-II receptor were investigated by thin-layer electro-
phoresis of an acid hydrolysate of the protein. Fig. 2
shows that incubation of plasma membranes from
control cells with [γ-32P]ATP resulted in the incorpora-
tion of [32P]phosphate into tyrosine residues. The
phosphoamino acid analysis of the receptor in mem-
branes derived from insulin-treated cells was complicated
by the very low amount of [32P]phosphate incorporated.
However, prolonged exposure of the thin-layer plates
revealed the presence of phosphotyrosine. No phos-
phoserine or phosphothreonine was detected. In a previous
paper [9] and in some of the experiments described
below, it was shown that the tyrosine kinase that
phosphorylates the IGF-II receptor is tightly bound to
the Triton X-100-insoluble fraction of the plasma
membrane, and that it preferentially phosphorylates the
IGF-II receptors contained within that fraction. Fig. 3

![Phosphorylation graph](https://example.com/fig3.jpg)

Plasma membranes were prepared from control cells (C)
or cells treated with 0.1 μM-insulin (I) as described in the
Materials and methods section. Plasma membranes were
resuspended in phosphorylation buffer containing 1%
Triton X-100. After 30 min at 5 °C, [γ-32P]ATP was
added, to a final concentration of 100 μM. The reactions
were terminated after incubation at 5 °C for the times
indicated (5, 7.5 and 10 min), by the addition of a 10-fold
excess of solubilizing buffer as described in the Materials
and methods section. The receptor immunoprecipitates
were applied on a 6%-polyacrylamide gel. Autoradi-
ographs of such gels showed one main band at M₉,
230000, which corresponds to the value for the IGF-II
receptor. Some of the radioactivity remained at the top of
the separating gel, possibly representing an aggregation
product of the solubilized immunoprecipitated receptor.
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Fig. 2. Phosphoamino acid analysis of the IGF-II receptor

Intact plasma membranes from control cells (C) or cells treated with 0.1 μM-insulin (I) were incubated with [γ-32P]ATP (25 μCi/μmol) as described in the Materials and methods section. The IGF-II receptor was immunoprecipitated from these membranes and phosphoamino acid analysis was performed as described in the Materials and methods section. The positions of the phosphoamino acid standards [phosphoserine, Ser(P), phosphothreonine, Thr(P) and phosphotyrosine Tyr(P)] are indicated. Exposure of the thin layers was 12 h (C) or 72 h (I).

shows that a large majority of the protein found in this preparation of adipocyte plasma membranes is soluble in 1% Triton X-100, whereas only few major proteins compose the Triton X-100-insoluble structure. Thus the activity of the tyrosine kinase toward the IGF-II receptor can be studied in the presence or absence of detergent-soluble membrane components, which might potentially regulate the phosphorylation reaction.

The results shown in Fig. 4 compare the magnitude of 32P incorporation into IGF-II receptors when the phosphorylation reaction was performed with (1) intact plasma membranes, (2) Triton X-100-solubilized plasma membranes, (3) the Triton X-100-insoluble fraction of the plasma membrane and (4) the Triton X-100-soluble fraction of the plasma membrane, all derived from control or insulin-treated cells. Several important findings were observed. First, as described previously [9], addition of Triton X-100 to the plasma membranes dramatically increased the incorporation of 32P into the IGF-II receptor (exposure times are indicated in the legend to Fig. 4).

Second, the effect of insulin to inhibit 32P incorporation into IGF-II receptors was most pronounced when the phosphorylation reaction was carried out with intact plasma membranes. On solubilization of the membranes in Triton X-100, the inhibitory effect persisted, but was less pronounced than that observed in intact membranes. However, when the Triton X-100-soluble components were removed from the insoluble fraction before initiation of the phosphorylation reaction, the inhibitory effect of insulin was completely abolished. Under these conditions the amount of 32P associated with the receptor band was actually increased in the receptors obtained from insulin-treated cells compared with controls. This effect could be accounted for by the increased number of receptors that exist in plasma membranes derived from insulin-treated cells [4,5]. Taken together, these results strongly suggest that the inhibition of 32P incorporation into IGF-II receptors observed in membranes from insulin-treated cells is conferred by a component of the plasma membrane which is soluble in Triton X-100.

Another observation made in this experiment is that 32P incorporation into the receptors present in the Triton X-100-soluble fraction of the plasma membrane could not be observed, neither when [γ-32P]ATP was added to the soluble portion after it was separated from the insoluble structure (Fig. 4) nor when the [γ-32P]ATP was added before separation of the fractions (results not shown). These results confirm that only the receptors present in the Triton-insoluble structure can become tyrosine-phosphorylated under the conditions of these experiments, perhaps reflecting a strict steric requirement for this reaction to occur. Analysis of the binding of 125I-IGF-II to these fractions revealed that approx. 20% of

Fig. 3. Protein composition of intact, Triton X-100-soluble and Triton X-100-insoluble fractions of adipocyte plasma membranes

Plasma membranes from control cells were isolated and electrophoresed on a 6–16% polyacrylamide slab gel. The left lane contains 50 μg of membrane protein. The middle lane is the fraction of this preparation that is soluble in 1% Triton X-100, and the right lane is the Triton X-100-insoluble material obtained from the same preparation. Positions of Mr standards are indicated.
The phosphorylation of the IGF-II receptor was studied in intact membranes (intact), in membranes resuspended in buffer containing 1% Triton X-100 (+ 1% Triton), in the Triton X-100-insoluble fraction of the membrane (Triton-insoluble), or in the Triton X-100-soluble fraction of the membrane (Triton soluble), all obtained from control (C) or insulin-treated (I) adipocytes. The phosphorylation buffer and the methods employed to obtain the different membrane fractions are described in the Materials and methods section. Phosphorylation reactions were started with 100 μM-[γ-32P]ATP, and continued for 10 min at 32°C. The IGF-II receptor was then immunoprecipitated, separated on a 6% polyacrylamide slab gel, and detected by autoradiography. The exposure time for the experiment carried out in intact plasma membranes was 72 h; for the rest of the experiments it was 20 h. Exposure of the gels for up to 5 days did not reveal 32P incorporation into receptors obtained from the Triton X-100-soluble membrane fraction. Similar results were obtained in five successive experiments, with different cell preparations.

The possibility that the Triton X-100-soluble component that mediates that inhibitory effect of insulin could be a phosphotyrosyl phosphatase activity was investigated. An experiment was performed in which a 10-fold excess of unlabelled ATP was added after incubation of the membranes with [32P]ATP. The activity of a receptor phosphotyrosyl phosphatase would be reflected, under these conditions, by a decrease in the amount of 32P in the receptor. The results of this experiment (Fig. 5) show that incubation of the membranes with unlabelled ATP for 10 min resulted in a small decrease in the amount of 32P in the IGF-II receptor, demonstrating the presence of a phosphotyrosyl phosphatase activity. However, the decrease was small, and no difference could be observed between membranes from control or insulin-treated cells. These results do not support the hypothesis that activation of a phosphotyrosyl phosphatase is responsible for the inhibitory effect of insulin. However, the possibility remains that the addition of a high concentration of ATP could cause a competitive inhibition of the enzyme [14] after the activities of other kinases or phosphatases that could be involved in the modulation of IGF-II-receptor phosphorylation. For this reason, a different experiment was performed in which Triton X-100-insoluble membrane fractions containing 32P-labelled IGF-II receptor were used as substrates to measure the phosphotyrosyl phosphatase activity of the Triton X-100-soluble membrane extracts from control or insulin-treated cells. No phosphatase activity could be observed on incubation of the 32P-labelled receptors with the Triton X-100-soluble extract from either control or insulin-treated cell membranes (Fig. 6). These results are consistent with those obtained by using an excess of unlabelled ATP to detect the presence of phosphotyrosyl phosphatase activity (Fig. 5), and suggest that under these conditions the activity of receptor phosphotyrosyl phosphatases is negligible.

It is important to note that all the experiments described above were performed in the presence of 80 mM-β-glycerophosphate and 10 mM-molybdate. Among several inhibitors tested (fluoride, zinc, β-glycerophosphate, vanadate and molybdate), these two were the most effective in promoting 32P incorporation into the IGF-II receptor.

It was decided to investigate the effect of these phosphatase-inhibitory agents on the effect of insulin to inhibit IGF-II-receptor phosphorylation in isolated membranes. The results shown in Fig. 7 compare the effects of molybdate, vanadate and fluoride on the phosphorylation of the IGF-II receptor in membranes from control and insulin-treated cells, and show that
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vanadate is almost as effective as molybdate in promoting the basal incorporation of [32P]phosphate into the receptor. Fluoride, a selective phosphoseryl phosphatase inhibitor, is much less effective in promoting IGF-II-receptor phosphorylation. Interestingly, whereas the decrease in IGF-II-receptor phosphorylation in membranes from insulin-treated cells persisted in the presence of vanadate, it became undetectable when fluoride was employed. Fluoride is known to have multiple effects on membrane components, such as the stimulation of adenylyl cyclase activity [15] and of photoreceptor phosphodiesterase [16], through its interaction with GTP-binding proteins [16]. However, an intriguing possibility is that fluoride could block the effect of insulin on tyrosine phosphorylation of the IGF-II receptor by inhibiting an insulin-sensitive phosphoserine phosphatase, which might in turn modulate the activity of the tyrosine kinase.

DISCUSSION

It has previously been shown that the receptor for IGF-II is phosphorylated in intact cells on serine and threonine residues [7,8]. In contrast, when isolated fat-cell plasma membranes are incubated with [γ-32P]ATP, the phosphorylation occurs principally on tyrosine residues on the receptor [9]. This phosphorylation reaction appears to be catalysed by a potent tyrosine kinase activity, which is tightly bound to the Triton X-100-insoluble portion of this membrane, and not from an autophosphorylation reaction [9]. Interestingly, the Triton X-100-insoluble fraction of several other cell types has been found to contain elevated tyrosine kinase activity [17-19], although phosphorylation of proteins on tyrosine residues occurs rarely in these cells. These findings suggest that the steady-state extent of tyrosine phosphorylation is greatly suppressed in intact cells, and is increased only under particular physiological circumstances or in response to specific stimuli.
Interestingly, removal of Triton X-100-soluble components of the plasma membrane greatly enhances the phosphorylation of the IGF-II receptor on tyrosine residues. Fig. 4 shows that phosphorylation of the receptor occurs to a much lesser extent in intact membranes than in Triton X-100-solubilized membranes. Separation of the Triton X-100-soluble components from the insoluble fraction enhances phosphorylation to an even greater extent.

A key finding in this paper is that tyrosine phosphorylation of the IGF-II receptor is dramatically inhibited in membranes derived from insulin-treated cells. The inhibitory effect of insulin cannot be observed when the Triton X-100-soluble proteins are removed from the insoluble material before the phosphorylation reaction (Fig. 4). Therefore, the effect of insulin can be due to an enhancement of the inhibitory interaction between one or more membrane components which are solubilized by the detergent, and the tyrosine kinase present in the non-soluble material.

Such an inhibitory component could be an insulin-sensitive phosphotyrosyl phosphatase. Removal of the detergent-soluble phosphatase would therefore eliminate the inhibitory effect of insulin. This possibility, however, is not supported by the experiments that have been performed. First, well-known phosphotyrosyl phosphatase inhibitors such as vanadate do not suppress the inhibitory effect of insulin (Fig. 7). Second, incubation of phosphorylated IGF-II receptors with unlabelled ATP (Fig. 5) or Triton X-100 extracts from control or insulin-treated membranes (Fig. 6) does not result in a marked dephosphorylation of the receptor, suggesting that IGF-II-receptor phosphotyrosine phosphatases are not active under the experimental conditions used.

An alternative possibility is that the effect of insulin may be indirectly mediated through the activation of a phosphoseryl/phosphothreonyl phosphatase. This possibility is raised by the finding that fluoride, which is a relatively selective inhibitor of such phosphatases, is the only compound which can completely suppress the inhibitory effect of insulin on IGF-II-receptor phosphorylation. Moreover, we have found that in intact cells, labelled with [32P]phosphate, insulin decreases the phosphorylation of the receptor on threonine and serine residues [7,8], suggesting that insulin may stimulate the activity of a phosphoseryl/phosphothreonyl phosphatase.

The physiological relevance of tyrosine phosphorylation of the IGF-II receptor is not known. However, it is interesting that both the phosphorylation of the receptor in vivo on serine and threonine residues, and the phosphorylation in vitro on tyrosine residues, are decreased in response to insulin. These results raise the possibility that similar mechanisms might be involved in mediating these effects. The enzymes involved in the phosphorylation of the receptor may be modulated in similar ways by insulin-sensitive kinases or phosphatases, or by the presence of stimulatory or inhibitory factors. The nature of the serine, threonine and tyrosine kinases that phosphorylate the IGF-II receptor, and of the insulin-sensitive components of the Triton X-100-soluble fraction, is at present unknown.

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