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A highly phosphorylated subpopulation of insulin-like growth factor II/mannose 6-phosphate receptors is concentrated in a clathrin-enriched plasma membrane fraction

(receptor recycling/insulin action/coated pits)

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ABSTRACT **Insulin-like growth factor II (IGF-II)/mannose 6-phosphate (Man-6-*P*) receptors immunoprecipitated from purified plasma membranes of ^{32}P-labeled rat adipocytes are markedly heterogenous in their phosphorylation state. Approximately 80% of the plasma membrane receptors are solubilized in 1% (vol/vol) Triton X-100 and are phosphorylated on serine residues at a stoichiometry of ≈0.1–0.2 mol of phosphate per mol of receptor. In contrast, 15–20% of the receptors are Triton X-100-insoluble and are phosphorylated on serine and threonine residues at ≈4 or 5 mol of phosphate per mol of receptor. This Triton X-100-insoluble membrane subfraction contains only 5% of the total plasma membrane protein and yet contains all of the clathrin heavy chain associated with plasma membrane, as detected by immunoblotting with a monoclonal antibody. Based on the relative yields of protein in the detergent-insoluble material, IGF-II/Man-6-*P* receptors are concentrated ≈3-fold in this clathrin-enriched subfraction. Insulin treatment of intact cells increased the total IGF-II/Man-6-*P* receptors in the Triton X-100-soluble fraction of the plasma membrane, whereas no change in receptor number in the detergent-insoluble fraction was seen. However, insulin markedly decreased the phosphorylation stoichiometry of the Triton X-100-insoluble receptors. Taken together, these results indicate that insulin decreases the phosphorylation state of a highly phosphorylated subpopulation of IGF-II/Man-6-*P* receptors on the plasma membrane. In addition, insulin action may prevent the concentration of these receptors in a clathrin-enriched membrane subfraction.**

Recently the receptor for insulin-like growth factor II (IGF-II) and the cation-independent receptor for mannose 6-phosphate (Man-6-*P*) have been discovered to be the same protein (1, 2). The well-known physiological functions of this receptor include targeting lysosomal enzymes from their sites of synthesis to the lysosomes and retrieval of those lysosomal enzymes that are secreted into the extracellular space (3). In addition, the IGF-II/Man-6-*P* receptor binds ^{125}I-labeled IGF-II with high affinity and mediates the internalization and degradation of this ligand (4). The receptor also binds IGF-I with low affinity but does not bind insulin. Furthermore, ligand-binding sites on this receptor for Man-6-*P* and IGF-II exhibit positive cooperativity (1). Whether or not the binding of IGF-II or Man-6-*P* to this receptor elicits any cellular response is at present controversial (5, 6).

Treatment of intact cells with insulin has been shown to cause a 3- to 5-fold stimulation of the binding and degradation of ^{125}I-labeled IGF-II (4, 7). This stimulatory effect of insulin is associated with a rapid increase in IGF-II/Man-6-*P* receptors on the cell surface (7, 8). Membrane fractionation experiments in isolated adipocytes showed that the rapid increase in the cell surface receptor number after insulin is due to a redistribution of receptors from a low-density microsomal membrane pool to the plasma membrane (9). IGF-II/Man-6-*P* receptors are phosphorylated on serine and threonine residues (10, 11). Receptors isolated from adipocyte plasma membranes are phosphorylated at a stoichiometry 2- to 3-fold higher than that of receptors from intracellular low-density microsomal membrane stores (11). The increased number of IGF-II/Man-6-*P* receptors on the plasma membrane in response to insulin coincides with a marked decrease in the phosphorylation stoichiometry of the plasma membrane receptors (11). These and other data suggested that the phosphorylation of plasma membrane IGF-II/Man-6-*P* receptors is important in targeting these receptors towards an endocytic pathway (11).

To further explore the role of IGF-II/Man-6-*P* receptor phosphorylation during transit of this receptor among cellular membranes, we sought to examine the phosphorylation state of the receptor at different points along the endocytic pathway. Ultrastructural studies have shown that the IGF-II/Man-6-*P* receptors on the plasma membrane are concentrated and internalized through clathrin-coated pits (12). Therefore, we looked for any difference in the phosphorylation of receptors associated with clathrin-coated pits and those in noncoated areas of the membrane. For this purpose, advantage was taken of the finding that clathrin-coated membranes resist solubilization by Triton X-100 (13).

In this paper we show that solubilization of purified adipocyte plasma membranes in Triton X-100 yields a membrane subfraction that is enriched in clathrin by ≈20-fold. This fraction contains ≈15% of the total plasma membrane IGF-II/Man-6-*P* receptors. These receptors are phosphorylated at a much higher stoichiometry than those receptors in the Triton X-100-soluble fraction of the plasma membrane. Furthermore, insulin causes a marked decrease in the phosphorylation of these receptors and their apparent exclusion from the clathrin-enriched, Triton X-100-insoluble membrane fraction. These experiments reveal that the phosphorylation state of IGF-II/Man-6-*P* receptors within the plasma membrane is strikingly heterogeneous and suggest that phosphorylation is involved in association of the receptor to membrane endocytic structures.

MATERIALS AND METHODS

Cell Isolation and Isotopic Labeling. Rat adipocytes were isolated from the epididymal fat pads of 75–100 g Sprague–Dawley rats by collagenase digestion (Worthington) in

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Abbreviations: IGF-II, insulin-like growth factor II; Man-6-*P*, mannose 6-phosphate; ^{125}I-IGF-II, ^{125}I-labeled IGF-II.

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Krebs–Ringer buffer with 25 mM Hepes, pH 7.4, containing bovine serum albumin at 30 mg/ml (Armour, Kankakee, IL) (14). Isolated cells (3×10^6) were incubated in 5 ml of this buffer containing 5 mCi/ml (1 Ci = 37 GBq) of [^{32}P]phosphate (carrier free, New England Nuclear) for 120 min at 37°C (11). Cells were treated without or with 10 nM insulin for 5 min at 37°C.

Plasma Membrane Isolation and Triton X-100 Solubilization. Cells were homogenized in an ice-cold buffer containing 0.25 M sucrose/10 mM Tris·HCl, pH 7.4/5 mM EDTA/1 mM phenylmethylsulfonyl fluoride/leupeptin at 20 μg/ml/50 mM sodium fluoride/50 mM sodium pyrophosphate/100 μM sodium vanadate. Plasma membranes were then isolated by differential centrifugation (4, 11), and resuspended to 2 mg/ml in an ice-cold buffer containing 10 mM Tris, pH 7.0/5 mM EDTA/1 mM phenylmethylsulfonyl fluoride/50 mM sodium fluoride. Triton X-100 was added to a final concentration of 1% by addition of an 11% (vol/vol) stock solution of the detergent at 1:10 (vol/vol). After 30 min on ice, the suspensions were centrifuged for 10 min at 12,000 × *g* in an Eppendorf microcentrofuge. The supernatant and the remaining pellet were separated and used as described below.

Immunoprecipitation of ^{32}P-Labeled Receptors. Intact plasma membranes or the Triton X-100-insoluble and -soluble fractions were dissolved in a lysis buffer containing 1.5% (vol/vol) Triton X-100, 1% (wt/vol) sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS)/1% bovine serum albumin/0.5 M sodium chloride and the protease and phosphatase inhibitors described above. IGF-II/Man-6-*P* receptors were then immunoprecipitated by adding 20 μg of a polyclonal antibody coupled covalently to Affi-Gel 10 (Bio-Rad) (11). After incubation of the membrane extracts, the resin was washed, and the receptor was separated by electrophoresis on 6% polyacrylamide slab gels as described (15). Gels were autoradiographed on Kodak X-Omat film at −70°C. The autoradiographs were used to localize the receptor band on the gels, and the radioactivity incorporated was measured by Cerenkov counting of the excised bands; background radioactivity in similarly sized gel pieces was subtracted.

Phosphoaminoacid Analysis. The gel pieces containing the IGF-II/Man-6-*P* receptor band were swollen in a buffer composed of 100 mM *N*-ethylmorpholine, pH 8.3. Trypsin (5 μg) was added, and digestion was done for 24 hr at 37°C. More than 95% of the radioactivity was eluted from the gel piece. The eluates were dried and hydrolyzed in 500 μl of 6 M HCl for 1–6 hr at 100°C. Electrophoresis was run on thin-layer cellulose plates (Brinkmann) for 120 min at 1000 V with a running buffer of acetic acid/pyridine/water (10:1:189).

Binding and Affinity Crosslinking of ^{125}I-Labeled IGF-II (^{125}I-IGF-II). IGF-II was purified from conditioned media of buffalo rat liver cells as described (16) and iodinated to a specific activity of 60 Ci/g using enzymobeads (Bio-Rad). Intact plasma membranes or the Triton X-100-insoluble membrane fractions were resuspended to a final concentration of 2 mg/ml in ice-cold Krebs–Ringer/Hepes buffer containing 0.2% bovine serum albumin. ^{125}I-IGF-II binding was measured as described (9). Alternatively, bis(sulfosuccinimidyl)suberate was added at a final concentration of 0.09 mM, and incubations were continued for 20 min. The reaction was stopped by adding 2 vol of 0.1 M Tris, pH 7.4. The membrane suspensions were centrifuged at 12,000 × *g* for 10 min, and the pellets were dissolved in Laemmli sample buffer containing 3% sodium dodecyl sulfate. The crosslinked receptors were separated by electrophoresis on polyacrylamide slab gels and measured by densitometric scanning of the autoradiograph from these gels.

Immunoblotting of Clathrin. Intact plasma membranes and the Triton X-100-soluble and -insoluble fractions from these membranes were prepared as described above. For dot-blotting, the Triton X-100-insoluble fractions were solubilized in 1% sodium deoxycholate, and solubilized proteins were applied onto nitrocellulose sheets. Alternatively, intact plasma membranes, or the Triton X-100-insoluble material from these membranes, were dissolved in Laemmli sample buffer containing 3% sodium dodecyl sulfate. The membrane polypeptides were separated by electrophoresis on polyacrylamide slab gels as indicated in each experiment. Immediately after electrophoresis, the proteins were electroeluted onto nitrocellulose paper (17) and probed with a monoclonal antibody (Boehringer Mannheim) against clathrin heavy chain sequences. The antibody was detected using a specific anti-mouse immunoglobulin coupled to alkaline phosphatase (Promega Biotec, Madison, WI) or a goat anti-mouse antibody and ^{125}I-labeled protein A (New England Nuclear).

Protein Assay. Membrane protein was measured using the procedure of Bradford (18) (Bio-Rad).

RESULTS

To examine the phosphorylation of IGF-II/Man-6-*P* receptors potentially associated with clathrin-coated structures of the plasma membrane a plasma membrane fraction enriched in such structures had to be obtained. To this end, plasma membranes were isolated from rat adipocytes in the presence of protease and phosphatase inhibitors and subsequently solubilized in 1% Triton X-100. Fig. 1 shows the polypeptide composition of intact adipocyte plasma membranes and the polypeptides soluble or insoluble in Triton X-100. Only a few membrane proteins are insoluble in this detergent under these experimental conditions. Quantitatively, such proteins compose only ≈5% of the total plasma membrane protein (data not shown). Strikingly, dot blotting of these fractions with monoclonal antibody against the heavy chain of clathrin indicated that the immunoreactive material exists exclusively in the Triton X-100-insoluble membrane fraction.

Immunoblotting of intact plasma membranes and of the Triton X-100-insoluble fraction recovered from an equal amount of membranes (Fig. 2) confirmed that the antibody reacted primarily with a 180-kDa polypeptide, which corresponds to the molecular weight of the clathrin heavy chain

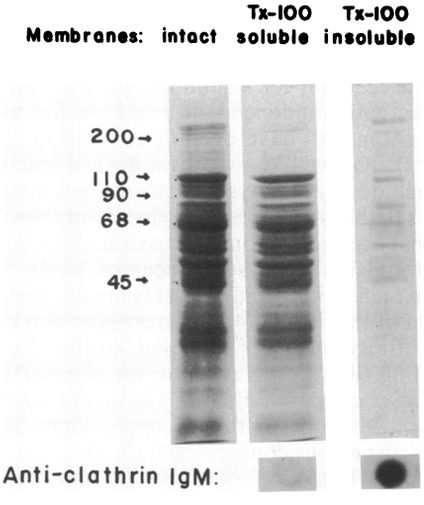

FIG. 1. Polypeptide composition of the Triton X-100-insoluble and -soluble fractions of the adipocyte plasma membrane. Membranes were isolated and solubilized in 1% Triton X-100 and separated on a 6–16% polyacrylamide gel. The gel was stained with Coomassie blue. Approximately 50 μg of Triton X-100-soluble protein and 5 μg of Triton X-100-insoluble protein were applied onto nitrocellulose paper and probed with anticlathrin monoclonal antibody and ^{125}I-labeled protein A. Autoradiographies of the blots are shown beneath the corresponding gel lanes.

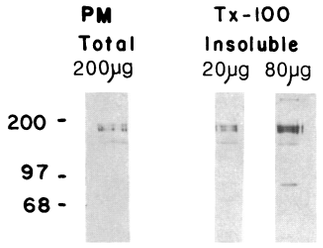

FIG. 2. Immunoblotting of isolated adipocyte plasma membranes (pM) and of the Triton X-100- (TX-100) insoluble fraction of the membrane with a monoclonal antibody against clathrin heavy chain. Plasma membranes were isolated and solubilized in Triton X-100. The indicated amounts of protein were separated on a 6% polyacrylamide minigel and electroeluted onto nitrocellulose paper. Blots were probed with anticlathrin heavy chain monoclonal antibody and a goat anti-mouse immunoglobulin coupled to alkaline phosphatase. Photographs of the developed blots are shown. The positions of prestained M_r markers are indicated.

molecule (19). Several lower-molecular weight bands were also detected. These bands apparently correspond to proteolytic fragments of the clathrin heavy chain, as their abundance increases markedly without protease inhibitors, whereas the 180-kDa polypeptide decreases proportionally under these conditions (data not shown). This experiment also indicates that the Triton X-100-insoluble fraction contains all detectable clathrin associated with the adipocyte plasma membrane (Fig. 2).

The abundance of IGF-II/Man-6-*P* receptors in the clathrin-enriched Triton X-100-insoluble fraction of the membranes was analyzed. Scatchard analysis of the binding of ^{125}I-IGF-II to plasma membranes or to the Triton X-100-insoluble fraction indicated that the receptors in both fractions bound IGF-II with similar affinity (K_d = 1 nM) (data not shown). Affinity-crosslinking experiments revealed that ≈15–20% of the receptors in the plasma membrane were found within the Triton X-100-insoluble fraction (Fig. 3, compare C lanes). Similar results were obtained when the abundance of IGF-II/Man-6-*P* receptors in the Triton X-100-insoluble fraction was measured by immunoblotting with the polyclonal antireceptor antibody and ^{125}I-labeled protein A (data not shown). Thus, under basal conditions IGF-II/Man-6-*P* receptors appear to be concentrated at least 3-fold within this fraction because this fraction composes only 5% of total plasma membrane protein. Treatment of cells with 10 nM

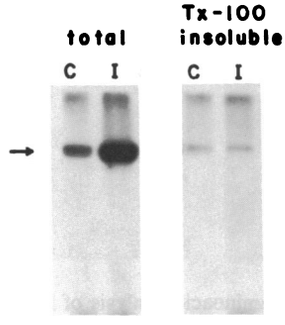

FIG. 3. Affinity crosslinking of ^{125}I-IGF-II to plasma membranes and to the Triton X-100-insoluble fraction of the membrane isolated from control or insulin-treated rat adipocytes. Plasma membranes were isolated from control (C) or insulin-treated (I) rat adipocytes and separated into two equal aliquots. One aliquot was solubilized in 1% (vol/vol) Triton X-100. Binding and crosslinking of 5 nM ^{125}I-IGF-II to the intact membranes and to the Triton X-100-insoluble membrane fractions obtained from such membranes was done as described. After crosslinking, the membranes were separated on a 6% polyacrylamide gel. Shown is an autoradiograph of the gel. This experiment was repeated five times with similar results.

insulin before fractionation caused a 3- to 5-fold increase in the number of IGF-II/Man-6-*P* receptors in the plasma membrane (Fig. 3, compare total lanes). Interestingly, the number of IGF-II receptors in the Triton X-100-insoluble membrane fraction did not increase upon insulin treatment. Thus, only 5% of the total IGF-II/Man-6-*P* receptors are present in the Triton X-100-insoluble fraction of plasma membranes from insulin-treated cells. As in controls, the Triton X-100-insoluble fraction of insulin-treated cell plasma membranes composes only 5% of the total protein. Thus, in insulin-treated cells the IGF-II/Man-6-*P* receptor does not appear to be concentrated in this plasma membrane fraction.

Immunoprecipitation of IGF-II/Man-6-*P* receptors from the Triton X-100-insoluble fraction of plasma membranes from cells labeled with [^{32}P]phosphate was done. These receptors contain a markedly greater amount of [^{32}P]phosphate compared with the receptors that are soluble in Triton X-100 (Fig. 4, compare C lanes). Cerenkov counting of the excised bands indicated that ≈90% of the receptor-associated [^{32}P]phosphate resides in the receptors from the Triton X-100-insoluble fraction (Table 1). That this difference in apparent phosphorylation state of the receptor between membrane subfractions might result from the phosphatase action in the Triton X-100-soluble fraction was considered. An experiment was done in which an aliquot of the Triton X-100-soluble fraction was mixed with an aliquot of the Triton X-100-insoluble material before immunoprecipitation. The ^{32}P amount in receptors from this mixture equaled the sum of the ^{32}P in the receptors from similar aliquots of the Triton X-100-soluble and -insoluble fractions immunoprecipitated independently (data not shown).

Treatment of cells with insulin markedly decreased the phosphorylation state of IGF-II/Man-6-*P* receptors within the Triton X-100-insoluble membrane fraction (Fig. 4, compare TX-100 insoluble lanes). The ^{32}P in the receptors from the detergent-soluble fraction is increased by insulin (Fig. 4, compare TX-100 soluble lanes). However, the number of receptors in this fraction is also increased in response to the hormone (Fig. 3). When the amount of ^{32}P is expressed as a function of receptor concentration, the stoichiometry of

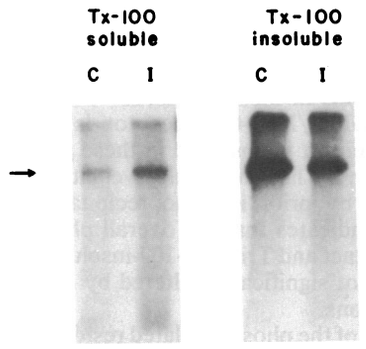

FIG. 4. ^{32}P-labeled IGF-II/Man-6-*P* receptor in the Triton X-100-soluble and -insoluble fractions of membranes from control or insulin-treated rat adipocytes. Isolated adipocytes were labeled by incubation with [^{32}P]phosphate for 2 hr and then treated without (C) or with (I) 10 nM insulin. Plasma membranes were isolated and solubilized in 250 μl of buffer containing 1% Triton X-100 as described. The Triton X-100-soluble and -insoluble fractions were separated, and the IGF-II/Man-6-*P* receptor was immunoprecipitated from each fraction. The immunoprecipitated receptor was separated on a 6% polyacrylamide gel and analyzed by autoradiography (≈30-min exposure). The arrow indicates the position of the receptor on the autoradiography. In some experiments, a small fraction of the receptor remained aggregated at the top of the stacking gel resembling an additional high-M_r band; the reason for this occasional aggregation is unclear. This experiment was repeated five times with similar results.

Table 1. Relative number and phosphorylation state of IGF-II/Man-6-*P* receptors obtained from the Triton X-100-insoluble or -soluble fractions of the adipocyte plasma membrane

Measurement	Triton X-100-insoluble		Triton X-100-soluble	
	Control	Insulin-treated	Control	Insulin-treated
[^{32}P]phosphate in IGF-II/Man-6-*P* receptors, cpm/band	3216	750	399	688
IGF-II/Man-6-*P* receptor [^{32}P]phosphate, % of total receptor-associated phosphate	89%	52%	11%	48%
^{125}I-IGF-II-binding, arbitrary units	1.18	0.93	7.0	19.3
^{125}I-IGF-II binding, % of total membrane binding	15%	4.6%	75%	95.4%
^{32}P/^{125}I	2725	806	57	35

Isolated adipocyte plasma membranes were obtained from [^{32}P]phosphate-labeled cells and solubilized in 1% Triton as described. The IGF-II/Man-6-*P* receptors were immunoprecipitated from the Triton-soluble or -insoluble fractions and separated by PAGE. The amount of ^{32}P in the receptor was quantified by Cerenkov counting of the excised bands. Results shown are the means of five experiments. For crosslinking experiments, membranes were obtained from unlabeled cells. ^{125}I-labeled IGF-II was bound and crosslinked to total membranes and to the Triton X-100-insoluble fraction from an equal amount of membranes. The crosslinked receptor was separated by PAGE, and measured by densitometric scanning of autoradiographs of the gels. The amount of receptor found in the Triton X-100 insoluble fraction was subtracted from the amount in the total membranes to estimate the amount of receptor in the soluble-membrane fractions.

phosphorylation of this receptor population apparently also decreases in response to insulin (Table 1, see ^{32}P/^{125}I).

The possibility that insulin might generally decrease the abundance or phosphorylation of the proteins that compose the Triton X-100-insoluble plasma membrane fraction was considered. To investigate this possibility, 2-μl aliquots of intact membrane suspensions or of Triton X-100-insoluble fractions from control or insulin-treated ^{32}P-labeled cells were analyzed by PAGE. Autoradiographs after brief (≈10-min) exposure of these gels revealed the most prominent phosphoproteins in these membrane fractions (Fig. 5). Because the IGF-II/Man-6-*P* receptor is a relatively minor phosphoprotein, it is obscured in these gels by major phosphoproteins in the high-molecular weight region and can only be analyzed by prior immunoprecipitation (Fig. 4). This experiment indicates that the overall phosphopeptide composition of intact and Triton X-100-insoluble adipocyte membranes was not significantly altered by insulin action under these conditions.

The nature of the phosphorylated residues on IGF-II/Man-6-*P* receptors from the Triton X-100-insoluble or -soluble fractions was investigated. The band corresponding to the receptors was excised from polyacrylamide gels, and the receptor was extracted as described. The extract was hydrolyzed for 3 hr in 6 M HCl and analyzed by thin-layer electrophoresis on cellulose plates. Autoradiographic analysis of the thin-layer plates indicated that the highly phosphorylated form of the receptor contained both phosphoserine and phosphothreonine residues (Fig. 6, TX-100 insoluble C lane). Interestingly, no phosphothreonine could be detected in either the receptors isolated from the Triton X-100-soluble membrane fraction or in the receptors obtained from insulin-treated cells (Fig. 6, other lanes). Phosphothreonine residues could not be detected in samples subjected to acid hydrolysis for <3 hr. Increasing the hydrolysis time to 6 hr or the time of autoradiography of the thin-layer plates revealed no previously undetected phosphothreonine in any samples.

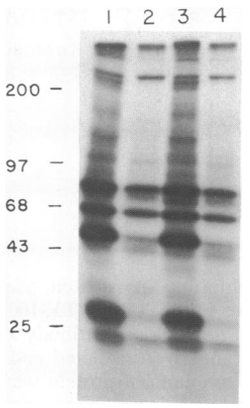

FIG. 5. Phosphopeptide composition of adipocyte plasma membranes and the Triton X-100-insoluble membrane fraction from control or insulin-treated rat adipocytes. Isolated adipocytes were labeled by incubation with [^{32}P]phosphate for 2 hr and then treated without (lanes 1 and 2) or with (lanes 3 and 4) 10 nM insulin. The plasma membranes were isolated from these cells and solubilized in 250 μl of 1% Triton X-100. Aliquots (2 μl) of intact plasma membranes (lanes 1 and 3) or of the Triton X-100-insoluble fraction from such membranes (resuspended to 250 μl) (lanes 2 and 4) were separated on a 6–16% polyacrylamide gel. Shown is an autoradiograph of the dried gel, exposed for 10 min. M_r markers at left; see text for discussion of bands.

DISCUSSION

The key finding reported in this paper is that the phosphorylation state of IGF-II/Man-6-*P* receptors in the plasma membrane of rat adipocytes is markedly heterogeneous. Approximately 80% of the receptors can be solubilized by Triton X-100. These receptors contain only ≈10% of the phosphate associated with the total population of plasma membrane IGF-II/Man-6-*P* receptors (Table 1). In contrast, ≈20% of the receptors are found to be insoluble in Triton

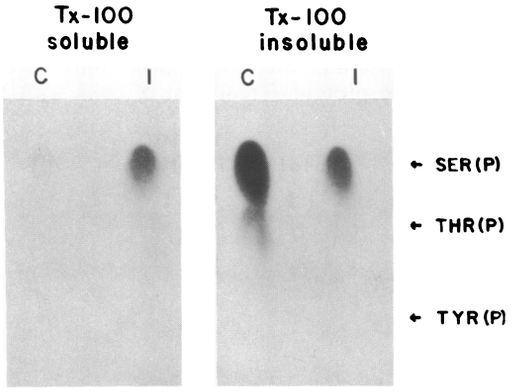

FIG. 6. Phosphoaminoacid analysis of IGF-II/Man-6-*P* receptors obtained from the Triton X-100 (TX-100)-soluble or -insoluble fractions of plasma membranes from control or insulin-treated rat adipocytes. Isolated adipocytes were labeled with [^{32}P]phosphate for 2 hr and then treated without (C) or with (I) 10 nM insulin. Plasma membranes were obtained and solubilized in Triton X-100. The IGF-II/Man-6-*P* receptor was immunoprecipitated from the Triton X-100-soluble or -insoluble fractions, separated by PAGE, extracted, and hydrolyzed as described. The phosphoaminoacids were separated by thin-layer electrophoresis. Shown is an autoradiograph of the thin-layer plate, exposed for 36 hr. The positions of the phosphoaminoacid standards are indicated. This experiment was repeated three times with similar results.

X-100. These receptors contain 90% of the total plasma membrane receptor-associated phosphate (Table 1). An average stoichiometry of about 1 mol of phosphate per mol of IGF-II-binding sites in the plasma membrane receptors has been previously estimated (11). Thus, the phosphorylation stoichiometry of most receptors (Triton X-100-soluble) must be ≈0.12 mol of phosphate per mol of IGF-II-binding sites. The highly phosphorylated form of the receptor (Triton X-100-insoluble) is phosphorylated to an approximate stoichiometry of 4 or 5 mol of phosphate per mol of IGF-II-binding site. In addition to the heterogeneity in the extent of receptor phosphorylation within the plasma membrane, a qualitative difference is also seen. The receptors obtained from the Triton X-100-insoluble membrane fraction contained both phosphoserine and phosphothreonine, whereas in those receptors obtained from the soluble fraction, only phosphoserine could be detected (Fig. 6).

A second important finding in these experiments is that the highly serine/threonine-phosphorylated form of the IGF-II/Man-6-*P* receptor resides in a membrane subfraction that contains virtually all the clathrin associated with adipocyte plasma membranes (Figs. 1 and 2). It has been shown that clathrin-coated structures and their associated proteins remain intact after exposure to Triton X-100 (13). Thus, the Triton X-100 insoluble fraction of the adipocyte plasma membrane probably contains clathrin-coated pits and their associated proteins. Biochemical or ultrastructural studies in adipocytes or other cell types indicate that the IGF-II/Man-6-*P* receptor exists in the plasma membrane and in intracellular membrane compartments adjacent to the Golgi region (5, 11, 20, 21). The receptors at the plasma membrane are concentrated in clathrin-coated pits, where they internalize and presumably recycle to the intracellular membrane compartments (12). In addition, the IGF-II/Man-6-*P* receptor has been found to associate *in vitro* with purified 50- to 100-kDa accessory coated-vesicle proteins and clathrin to form coated cages (22). Such structures form in the absence of added lipids at a stoichiometry of one receptor to one 100-kDa polypeptide to one clathrin heavy chain (22). Taken together, these data suggest that the IGF-II/Man-6-*P* receptor found within the Triton X-100-insoluble plasma membrane fraction may be concentrated in clathrin-coated pits. However, our data do not exclude the possibility that the receptor may be associated with other detergent-insoluble components, such as cytoskeletal proteins.

Clathrin-coated structures have been shown to contain several protein kinase activities (23–25). One of these protein kinases has been identified as casein kinase II (25). This enzyme phosphorylates clathrin β light chain to a high stoichiometry, as well as exogenous substrates added to coated structures (25). The purified IGF-II/Man-6-*P* receptor can be phosphorylated by purified casein kinase II on a tryptic peptide that comigrates on two-dimensional peptide mapping with the main tryptic peptide phosphorylated on the receptor *in vivo* (26). In addition, the deduced amino acid sequence of the cytoplasmic portion of the receptor has revealed several highly conserved serine and threonine residues flanked by acidic regions that compose typical casein kinase II phosphorylation sites (1, 2). The data of this paper are consistent with the hypothesis that casein kinase II or similar enzyme may be confined to clathrin-coated regions on the plasma membrane and may phosphorylate those IGF-II/Man-6-*P* receptors within these structures. The physiological consequences of this phosphorylation reaction are unknown, but the reaction could serve to anchor the receptor to the coated pit or to promote subsequent endocytosis.

Our present data confirm our previous finding that insulin treatment decreases the phosphorylation of IGF-II/Man-6-*P* receptors in the plasma membrane (11, 26). The present data reveal that this decrease occurs on both serine and threonine residues. In addition, in plasma membranes from insulin-treated cells, IGF-II/Man-6-*P* receptors are not concentrated in the clathrin-enriched Triton X-100-insoluble membrane fraction (Fig. 3). These results may indicate that the Triton X-100-insoluble membrane fraction is saturated with receptors under basal conditions and that further concentration of additional receptors present in insulin-treated cell membranes may not be possible. Alternatively, insulin treatment may alter IGF-II/Man-6-*P* receptor recycling in a way that prevents the concentration of receptors in membrane endocytic structures. For example, if receptor phosphorylation were necessary for its interaction with coated pit components, activation of a receptor phosphatase or inhibition of a kinase by insulin could prevent this interaction. Another possibility is that insulin may exclude the receptor from regions of the membrane that contain the IGF-II/Man-6-*P* receptor kinases—secondarily resulting in decreased receptor phosphorylation. Whether direct mechanistic relationships exist between IGF-II/Man-6-*P* receptor phosphorylation and its membrane localization in control or insulin-treated cells will have to be resolved in future work.

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