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Insulin-Induced Redistribution of the Insulin-Like Growth Factor II/Mannose 6-Phosphate Receptor in Intact Rat Liver

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Abstract The ability of acute insulin treatment to elicit a redistribution of the liver insulin-like growth factor-II/mannose 6-phosphate (IGF-II/M6P) receptor has been studied in rats, using cell fractionation. Injection of insulin (0.4–50 μg) led to a time- and dose-dependent decrease in IGF-II binding activity in Golgi-endosomal (GE) fractions, along with an increase in activity in the plasma membrane (PM) fraction; only receptor number was affected. Quantitative subfractionation of the microsomal fraction on sucrose density gradients showed that IGF-II binding activity distributed similarly to galactosyltransferase (a Golgi marker), at slightly higher densities than in vivo internalized ^{125}I-insulin, and at lower densities than 5' nucleotidase and alkaline phosphodiesterase (two plasma membrane markers). Insulin treatment led to a slight time-dependent and reversible shift of IGF-II binding activity toward higher densities. Subfractionation of the GE fraction on Percoll gradients showed that IGF-II binding activity was broadly distributed, with about 60% at low densities coinciding with galactosyltransferase and early internalized ^{125}I-insulin and with 40% at high densities in the region of late internalized ^{125}I-insulin. Insulin treatment caused a time-dependent and reversible shift of the distribution of IGF-II binding activity toward low densities. On SDS-PAGE, the size of the affinity-labeled IGF-II/M6P receptor was comparable in GE and PM fractions (about 255 kDa), but on Western blots receptor size was slightly lower in the latter (245 kDa) than in the former (255 kDa). Insulin treatment did not affect the size, but modified the abundance of the IGF-II/M6P receptor in a manner similar to that of IGF-II binding. In vivo chloroquine treatment fully suppressed the changes in IGF-II binding activity in liver GE and PM fractions observed in insulin-treated rats. We conclude that insulin elicits a time-dependent and reversible redistribution of liver IGF-II receptors from Golgi elements and endosomes to the plasma membrane, presumably via early endosomes. J. Cell. Biochem. 77:310–322, 2000.

Key words: insulin; insulin-like growth factor II; insulin-like growth factor II/mannose 6-phosphate receptors; liver; Golgi apparatus; endosomes; plasma membrane

The IGF-II/mannose 6-phosphate (IGF-II/M6P) receptor is a multifunctional membrane protein localized mainly in the Golgi and endosomes, and to a lesser extent at the cell surface [reviewed by von Figura and Hassilik, 1986; Kornfeld, 1992]. In the Golgi apparatus, this receptor binds newly synthesized lysosomal hydrolases bearing mannose 6-phosphate (M6P) moieties on their carbohydrate side chains and mediates their transport to endosomes. At the plasma membrane, the IGF-II/M6P receptor binds extracellular IGF-II and mannose 6-phosphate containing proteins, such as transforming growth factor-β (TGF-β) and proliferin, and mediates their endocytosis. A more limited role of the plasma membrane receptor in mediating some effects of IGF-II via G proteins has also been reported.

Structurally, the IGF-II/M6P receptor is a type I transmembrane glycoprotein of 260 kDa that contains a long extracellular (intraluminal) domain, a single transmembrane domain, and a short cytoplasmic domain [reviewed by Kornfeld, 1992]. Both IGF-II and mannose 6-phosphate-containing ligands bind to the extracellular domain, albeit at distinct sites. Soluble forms of the IGF-II/M6P receptor, generated by proteolytic cleavage of the membrane receptor, have been identified in serum [Causin et al., 1988; Kiess et al., 1987; McDonald et al., 1989], urine [Causin et al., 1988], and media of

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cultured tissues and cells [Clairmont and Czech, 1991; Bobek et al., 1991, 1992]. These soluble forms lack the transmembrane and cytoplasmic domains of the membrane receptor but retain the ability to bind IGF-II and M6P-containing proteins.

Studies on various cell types have shown that, under basal conditions, the IGF-II/M6P receptor cycles constitutively between the Golgi, the endosomes, and the plasma membrane [reviewed by Kornfeld, 1992]. However, at least in adipocytes [Oppenheimer et al., 1983; Oka et al., 1984; Wardzala et al., 1984] and skeletal muscle cells [Zhou et al., 1998], intracellular IGF-II/M6P receptors undergo rapid, reversible redistribution to the cell surface in response to physiological concentrations of insulin. Another effect of insulin, when injected in rats in vivo, is to induce an increase in the level of the truncated soluble IGF-II/M6P receptor in serum [Clairmont and Czech, 1990]. As the liver is the main contributor to the serum receptor in adult rats [Bobek et al., 1991, 1992], it is likely that, in vivo, insulin induces a redistribution of liver intracellular receptors to the cell surface, accompanied or followed by a limited proteolysis. To address this question, the effect of insulin treatment on the subcellular distribution and molecular size of the hepatic IGF II/M6P has been examined in rats, using cell fractionation techniques.

MATERIALS AND METHODS

Chemicals

Porcine insulin was purchased from Novo-Nordisk. Human recombinant IGF-II was obtained from Upstate Biotechnology, and natural IGF-II was extracted and purified from human placenta, as described by de Ceuninck et al. [1995]. Polyclonal antibody against rat placental IGF-II receptor was a kind gift of Dr. M.P. Czech and Ms. J. Buxton (Department of Biochemistry, University of Massaschussets Medical Center, Worcester, MA). Nycodenz was from Nycomed Pharma. Carrier-free ^{125}I, Percoll, and Protein A-Sepharose were from Amersham-Pharmacia. Chloroquine diphosphate, radioimmunoassay (RIA)-grade bovine serum albumin (BSA), protein A, 1,10 phenanthroline, bacitracin, leupeptin, aprotinin, benzamidine, and phenylmethylsulfonyl fluoride (PMSF) were from Sigma-Aldrich. Reagents for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and nitrocellulose membranes were from Bio-Rad. Kodak X-Omat A5–5 films were from Eastman Kodak.

Animals and Injections

Male Sprague-Dawley rats (body weight, 180–200 g) were obtained from Charles River France. After fasting for 16 h, rats received a single intravenous injection of porcine insulin (0.4–50 μg) diluted in 0.15 M NaCl under light ether anesthesia; uninjected rats or saline-injected rats were used as controls. At the indicated times, a blood sample was withdrawn from the orbitary plexus, using a heparinized Pasteur pipette; the liver was removed from the abdominal cavity through a median incision and immediately homogenized. When indicated, rats received two intraperitoneal injections of chloroquine dissolved in 0.15 M NaCl (4 mg/100 g body weight) at 2 h and 1 h before removal of the liver [Posner et al., 1982]. In studies designed to label early and late endosomes, ^{125}I-labeled insulin (about 2×10^6 cpm) was intravenously injected at 90 s and 8 min before killing, respectively.

Liver Subcellular Fractionation

Liver subcellular fractionation was performed using standard preparative and analytical procedures with minor modifications. 1,10-Phenantroline (2.5 mM), bacitracin (0.2 mg/ml), leupeptin (5 μg/ml), pepstatin (2.5 μg/ml), aprotinin (20 μg/ml), benzamidine (1 mM), and PMSF (0.2 mM) were routinely included in the homogenization and fractionation media. The microsomal fraction was prepared from homogenates in 0.25 M sucrose by differential centrifugation. Light, intermediate, and heavy Golgi-endosomal fractions were isolated separately or in combination from the microsomal fraction by discontinuous density gradient centrifugation, using a modification [Desbuquois et al., 1982] of the method of Ehrenreich et al. [1973]. A plasma membrane fraction was isolated from liver homogenates in 1 mM CO_3NaH according to the method of Neville [1968] up to step 11. In some experiments, the microsomal fraction was subjected to quantitative, analytical subfractionation on linear sucrose and Nycodenz density gradients. Sucrose gradients (density range, 1.04–1.25 g-cm^{-3}) were centrifuged for 15 h at 27,000 rpm in a Beckman SW 28 rotor and Nycodenz gradients (density range, 1.04–

1.16 g-cm^{-3}) were centrifuged for 1 h at 40,000 rpm in a Beckman SW 41 rotor. In each case, about 20 fractions were collected and analyzed for density by refractometry. The combined light and intermediate Golgi-endosomal fraction was subfractionated on self-generated Percoll density gradients (17.2%, v/v) in 0.25 M sucrose as described by Khan et al. [1982]. Cell fractions were stored at −80°C until analyzed. In preparative procedures, results of biochemical determinations were expressed relatively to cell fraction protein (specific activity) or liver weight (recovery). In analytical procedures, results were related to density (sucrose and Nycodenz gradients) or fraction number (Percoll gradients) and presented as normalized histograms. Median densities were derived from these experiments as described by Beaufay et al. [1964]. Calculations were made using an LCIII Macintosh computer.

Preparation of Radioiodinated Ligands and Measurement of IGF-II Binding to IGF-II/M6P Receptors

^{125}I-labeled IGF-II (75–150 μCi/μg), ^{125}I-labeled insulin (80–120 μCi/μg), and ^{125}I-labeled protein A (20 μCi/μg) were prepared using chloramine T and purified by gel filtration on Sephadex G-50.

Binding of ^{125}I-labeled IGF-II to liver subcellular fractions was measured as described by Scott and Baxter [1987], with minor modifications. Incubation mixtures contained, in 0.2–0.3 ml of 25–50 mM Tris HCl buffer, pH 7.4, 0.1% Triton X-100, 2.5 mg/ml BSA, 0.5 mg/ml bacitracin, 5 mM $MgCl_2$, 0.5–10 μg cell fraction protein, and 6,000–12,000 cpm ^{125}I-labeled IGF-II. After 3 h at 23°C or 16 h at 4°C, receptor-bound IGF-II was precipitated by polyethyleneglycol (PEG) (12.5%, w/w) in the presence of bovine γ-globulin (0.5 mg) as a carrier. Binding of IGF-II to liver cell fractions was corrected for nonspecific binding (6–8% of the total radioactivity added) as measured in parallel incubations in which excess native IGF-II was added or cell fractions were omitted.

Affinity Cross-Linking of ^{125}I-Labeled IGF-II to the IGF-II/M6P Receptor and SDS-PAGE Analysis of the Cross-Linked Complex

Subcellular fractions (25–50 μg protein) were incubated with 100,000 cpm ^{125}I-labeled IGF-II in 0.2 ml of 0.2 M phosphate buffer, pH 7.4, for 150 min at 23°C. Dissucimidyl suberate (0.3 mM) was added and, after 15 min, the reaction was terminated by the addition of 10 mM Tris HCl buffer, pH 7.4, containing 1 mM EDTA. Whole incubation mixtures or membranes derived by centrifugation were supplemented with SDS (2%, v/v) and β-mercaptoethanol (2%, v/v), boiled for 3 min, and subjected to polyacrylamide gel electrophoresis (3% stacking gel and 5% resolving gel) [Laemmli, 1970]. Standard molecular-weight markers (filamin, 250 kDa; myosin, 200 kDa) were subjected to electrophoresis in one lane of the gel along with cell fractions. Gels were stained with Coomassie blue and were destained, dried, and exposed to Kodak X-Omat AR-5 films in cassettes equipped with intensifying screens. The molecular weight of radiolabeled proteins identified on autoradiograms was determined by comparison to the mobility of standard proteins.

Western Immunoblotting of IGF-II/M6P Receptors

Subcellular fractions (50–300 μg protein) were subjected to reducing SDS-PAGE as described above. Proteins were electrotransferred to a nitrocellulose membrane for 4 h at 440 mA in 25 mM Tris, 192 mM glycine buffer, pH 8.3, containing 0.1% SDS. The membrane was incubated first in NaCl/phosphate buffer (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 and 1.7 mM KH_2PO_4, pH 7.4) containing 0.1% Tween 20 and 1% (w/v) BSA for 1 h at 20°C, and then in the same solvent containing antibody against the IGF-II/M6P receptor for 16 h at 4°C (1:1,000). After washing with NaCl/phosphate buffer 0.1% Tween 20 at 20°C, the membrane was incubated for 1 h at 23°C with ^{125}I-labeled protein A, washed again, and subjected to autoradiography. The radiolabeled bands were quantitated by scanning densitometry (Hewlett Packard, ScanJet II scanner connected to a MacIntosh computer) and their molecular weight determined as described above.

Assay of the Soluble IGF-II/M6P Receptor in Blood Plasma

After removal of blood cells by centrifugation, plasma was diluted 1:3 into 0.1 M phosphate buffer containing 1 mg/ml bacitracin, 500 U/ml aprotinin, 5 μg/ml leupeptin, and 0.2

mM PMSF; 0.3–0.5 ml of this dilution was subjected to centrifugation on 10-ml linear sucrose gradients (5–20%, w/v, density range 1.015–1.075 g-cm^{-3}) for 20 h at 200,000*g* [Hardouin et al., 1987]. Under these conditions, the soluble IGF-II/M6P receptor was recovered in subfractions of density above 1.065 g-cm^{-3} as judged on affinity cross-linking with ^{125}I-IGF-II, Western immunoblotting, and immunoprecipitation of the ^{125}I-IGF-II-receptor complex, using protein A-Sepharose, whereas other IGF binding proteins were recovered at lower densities (results not shown). The soluble receptor in dense gradient fractions was assayed for ^{125}I-labeled IGF-II binding followed by PEG precipitation as described for the membrane receptor.

Other Biochemical Assays

Protein [Lowry et al., 1951], galactosyltransferase [Beaufay et al., 1974], 5' nucleotidase [Aronson and Touster, 1974], and alkaline phosphodiesterase [Aronson and Touster, 1974] in liver cell fractions were measured according to standard procedures.

Statistical Methods

Comparisons between experimental groups were carried out using Student's *t*-test and the nonparametric Kruskal-Wallis test.

RESULTS

IGF-II Binding Activity in Microsomal, Golgi-Endosomal, and Plasma Membrane Fractions

The subcellular distribution of liver IGF-II binding activity was first examined using preparative procedures. Cell fractions selected for study included the microsomal fraction, which contained 90 ± 6% (mean ± SEM, n = 4) of total sedimentable IGF-II binding activity, three Golgi-endosomal fractions of different densities, and a plasma membrane fraction. When expressed relatively to cell fraction protein, IGF-II binding activity in control rats was about sixfold higher in Golgi-endosomal fractions and fourfold lower in the plasma membrane fraction than in the microsomal fraction (Fig. 1, top). However, because of their low protein content, the three Golgi-endosomal fractions accounted together for only 8% of the binding activity associated with the microsomal fraction and recovery of activity in the plasma membrane fraction was about 0.5% (Fig. 1, bottom).

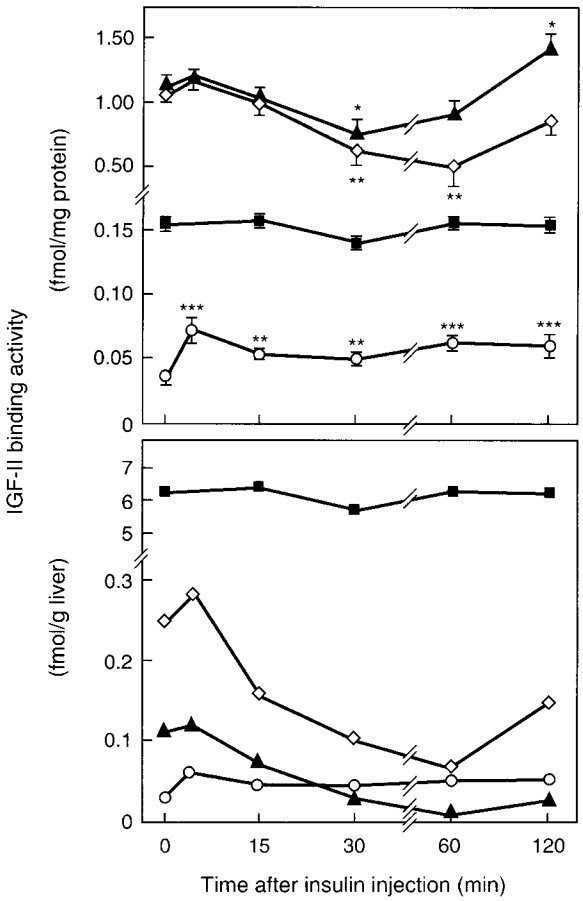

Fig. 1. Changes in IGF-II binding activity of liver cell fractions at various times after insulin injection. Rats were killed at the indicated times after injection of 50 μg insulin. Liver microsomal (), plasma membrane (○) and light (◇) and intermediate (▲) Golgi-endosomal fractions were prepared and assayed for IGF-II binding activity, with results expressed as specific activity (top) and recovery (bottom). Recoveries were calculated by multiplying average specific activities by average protein yields. Details of the procedures are given under Materials and Methods. The results shown are the mean ± SEM of five to eight individual determinations on separate livers. Asterisks indicate a statistically significant difference between control and insulin-treated rats (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).

Injection of 50 μg insulin, a dose that ensures a nearly maximal occupancy of liver insulin receptors [Desbuquois et al., 1982] led to a time-dependent increase in IGF-II binding activity in the plasma membrane fraction, along with a decrease in binding activity in the light and intermediate Golgi-endosomal fractions. Binding activity was not significantly affected in either the heavy Golgi-endosomal

fraction or the total microsomal fraction. When related to cell fraction protein (Fig. 1, top), the changes in IGF-II binding activity induced by insulin were maximum at 4 min in the plasma membrane fraction (twofold increase), 30 min in the intermediate Golgi-endosomal fraction (40% decrease), and 60 min in the light Golgi-endosomal fraction (twofold decrease). These changes were still detectable at 2 h in the plasma membrane and in the light Golgi-endosomal fraction but were no longer observed in the intermediate Golgi fraction. When related to liver weight (Fig. 1, bottom), the insulin-induced increase in IGF-II binding activity in the plasma membrane fraction was of similar magnitude. However, because of an insulin-induced decrease in protein recovery in the light and intermediate Golgi-endosomal fractions, the decrease in IGF-II binding activity in these fractions was of greater magnitude and of longer duration, reaching 80% at 1 h and still detectable at 2 h in both fractions.

The insulin-induced changes in IGF-II binding activity in the Golgi-endosomal and plasma membrane fractions were dose dependent. In rats killed 30 min after insulin injection, these changes were detectable at 2 μg in the light Golgi-endosomal fraction and 10 μg in the intermediate Golgi-endosomal fraction and the plasma membrane fraction (Fig. 2).

Scatchard plots constructed from competition studies with unlabeled IGF-II showed that, regardless of the fraction, only receptor number was modified by insulin treatment, affinity remaining unchanged (Table I). In rats killed 60 min after insulin injection, IGF-II/M6P receptor number was decreased sixfold in the light Golgi-endosomal fraction and twofold in the intermediate Golgi-endosomal fraction and was increased twofold in the plasma membrane fraction.

The insulin-induced changes in IGF-II/M6P receptor number in liver cell fractions were accompanied by an increase in soluble IGF-II/M6P receptor level in blood plasma, as judged on IGF-II binding activity migrating at the position of the receptor on sucrose gradients. When expressed as percentage increase above control (no insulin treatment), this change was 42 ± 4 at 4 min, 48 ± 5 at 15 min, 43 ± 3 at 30 min, 29 ± 3 at 1 h, and 36 ± 3 at 2 h (mean ± SEM, 5–7 determinations).

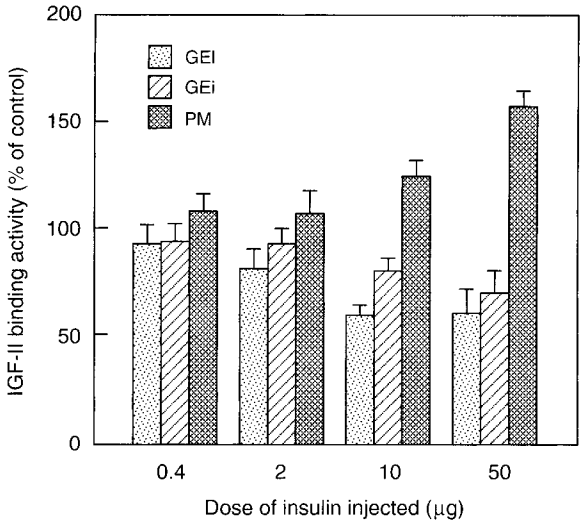

Fig. 2. Changes in IGF-II binding activity of liver cell fractions as a function of the dose of insulin injected. Rats were killed 30 min after injection of insulin at the indicated dose. Liver subcellular fractions were prepared and assayed for IGF-II binding activity as described under Materials and Methods. The results are expressed as the percentage of IGF-II binding activity in control rats: mean ± SEM of five determinations (50-μg dose) or the mean ± one-half the range of duplicate determinations (other doses).

Distribution of the IGF-II Binding Activity Associated With the Microsomal Fraction on Analytical Density Gradients

Although suggesting an insulin-induced redistribution of the IGF-II/M6P receptor, the results described were obtained using cell fractions that account for less than 10% of total cellular receptors. In addition, the plasma membrane fraction contains membranes that originate to a large extent from the lateral and contiguous domains of the hepatocyte [Neville, 1968]. Accordingly, the microsomal fraction, which contains 90% of total cellular IGF-II/M6P receptors and is more enriched in membranes originating from the hepatocyte sinusoidal domain [Wisher and Evans, 1975], was quantitatively subfractionated on analytical sucrose and Nycodenz gradients. The density distribution of IGF-II binding activity was compared with that of protein and of the following markers: galactosyltransferase (Golgi); in vivo injected ^{125}I-insulin taken up into the liver at 90 s and 8 min (early and late endosomes, respectively); and 5' nucleotidase and alkaline phosphodiesterase (plasma membranes).

On sucrose gradients (Fig. 3 and Table II), IGF-II binding activity in control rats showed a

TABLE I. Scatchard Analysis of IGF-II Binding to Liver Subcellular Fractions of Control and Insulin-Treated Rats*

	K_d (10^{-10} M)				R (pmol/mg protein)			
Treatment	GEl	GEi	GEh	PM	GEl	GEi	GEh	PM
No treatment	0.72	0.59	1.28	0.22	11.4	12.3	10.4	0.30
Insulin treatment								
30 min	0.63	0.63	1.73	—	4.18	5.70	9.04	—
60 min	0.52	0.71	1.32	0.35	1.77	7.15	10.2	0.66
120 min	0.83	0.61	1.27	0.23	8.83	11.9	10.9	0.57

*Plasma membranes (PM) and light (GEl), intermediate (GEi), and heavy (GEh) Golgi-endosomal fractions from untreated rats and rats killed at the indicated time after insulin injection (50 μg) were examined for ^{125}I-labeled IGF-II binding in the presence of serial concentrations of native IGF-II. The results were plotted according to Scatchard and analyzed using a one-site model.

symmetrical, unimodal distribution, with a median density of 1.127 g-cm^{-3}. This distribution was closely similar to that of galactosyltransferase (median density, 1.125 g-cm^{-3}) and markedly overlapped with that of ^{125}I-insulin, which migrated at slightly lower densities (median densities, 1.120 g-cm^{-3} at 90 s and 1.109 g-cm^{-3} at 8 min). It clearly differed, however, from the distribution of 5' nucleotidase, alkaline phosphodiesterase, and protein, which were recovered at higher densities (median densities, about 1.15–1.17 g-cm^{-3}). In rats killed 4 min and 15 min after insulin injection, a slight but reproducible shift of IGF II binding activity toward higher densities was observed, resulting in an increased median density. Later, this shift was no longer detectable, and median densities did not differ from that observed in control rats.

On Nycodenz gradients, the distribution of IGF-II binding activity also overlapped to a large extent with the distribution of galactosyltransferase and of ^{125}I-insulin, which were recovered at slightly higher densities; the distribution differed from that of 5' nucleotidase, which was recovered at higher densities (results not shown and Table II). As with sucrose gradients, insulin treatment led to a slight shift of IGF-II binding activity toward higher densities, which was detectable at 4 and 15 min. Taken together, these findings indicate that insulin treatment induces a rapid and reversible translocation of the IGF-II/M6P receptor from Golgi and/or endosomal components to components equilibrating at a higher density, presumably plasma membranes.

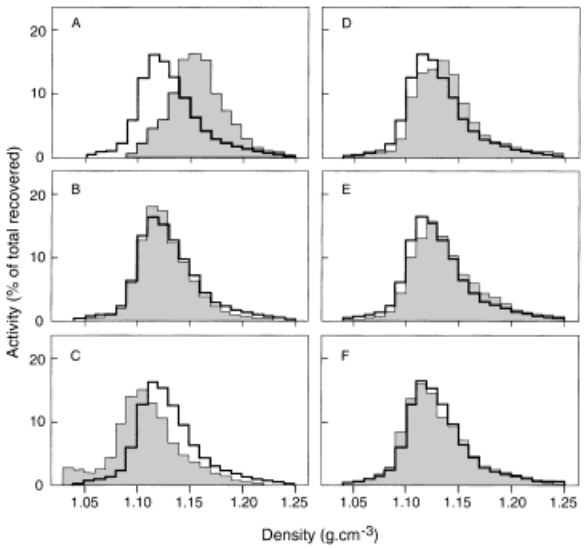

Fig. 3. Distribution of IGF-II binding activity associated with the microsomal fraction of control and insulin-treated rats on sucrose gradients. Microsomal fractions from control and insulin-injected rats were prepared and subfractionated on linear sucrose density gradients as described under Materials and Methods. Subfractions were assayed for IGF-II binding, galactosyltransferase, and in vivo uptake of ^{125}I-insulin, with results expressed as percentage of total recovered per normalized density intervals of 0.01 g-cm^{-3}. Left: distribution of IGF-II binding activity in control rats (clear histograms, thick line) compared with the distribution of various markers (dark histograms, thin line): 5' nucleotidase (**A**); galactosyltransferase (**B**); and uptake of ^{125}I-insulin at 8 min (**C**). Right: distribution of IGF-II binding activity in rats killed 4 min (**D**), 15 min (**E**), and 120 min (**F**) after insulin injection (dark histograms, thin line) compared with the distribution of binding activity in control rats (clear histograms, thick line). The results shown are the mean of at least three separate experiments on separate livers, except for ^{125}I-insulin uptake and IGF-II binding activity at 4 min, the mean of two experiments. The median densities derived from these experiments are shown in Table II.

TABLE II. Median Densities for IGF-II Binding Activity and Other Constituents Upon Centrifugation of the Microsomal Fraction on Sucrose and Nycodenz Gradients*

	Sucrose gradients	Nycodenz gradients
IGF-II binding activity		
No treatment	1.127 ± 0.002 (7)	1.080 ± 0.004 (5)
Insulin treatment		
90 s	1.128 ± 0.001 (3)	1.083 ± 0.001 (2)
4 min	1.135 ± 0.001 (2)	1.084 ± 0.002 (2)
15 min	1.131 ± 0.001 (3)	1.084 ± 0.002 (3)
60 min	1.125 ± 0.003 (3)	1.082 ± 0.001 (4)
120 min	1.125 ± 0.006 (3)	1.082 ± 0.005 (3)
Galactosyltransferase	1.125 ± 0.001 (3)	1.083 ± 0.002 (5)
In vivo ^{125}I-insulin uptake		
90 s	1.120 ± 0.001 (2)	1.088 ± 0.001 (2)
8 min	1.109 ± 0.002 (2)	1.082 ± 0.001 (2)
5′ Nucleotidase	1.156 ± 0.003 (3)	1.115
Alkaline phosphodiesterase	1.168 ± 0.002 (2)	—
Protein	1.164 ± 0.002 (6)	1.104 ± 0.004 (8)

*The values indicated (expressed in g-cm^{-3}, mean ± SD of the indicated number of determinations) have been determined as described under Materials and Methods. The density-distribution plots from which these values are derived are shown in part in Figure 3.

Distribution of IGF-II Binding Activity Associated With Golgi-Endosomal Fractions on Percoll Density Gradients

The Golgi-endosomal fractions as isolated in this study were heterogeneous and contained both Golgi elements and endosomes. In an attempt to resolve these components, a combined light and intermediate Golgi-endosomal fraction was subfractionated on Percoll density gradients, and the distribution of IGF-II binding activity was compared with that of galactosyltransferase and in vivo ^{125}I insulin uptake (Fig. 4). In control rats, IGF-II binding activity showed a broad distribution with about 60% in the upper half of the gradient, coinciding with galactosyltransferase and ^{125}I-insulin taken up at early time, and 40% in the lower half, coinciding with ^{125}I-insulin taken up at late time. Injection of insulin led to a reproducible, time-dependent shift of the distribution of IGF-II binding activity from the lower to the upper region of the gradient. This shift was maximal at 15 min, still detectable at 30 min, but no longer observed after 60 min. At 120 min, an opposite shift of IGF-II binding activity toward the high density region of the gradient was even observed. These findings suggest that the insulin-induced redistribution of the IGF-II receptor to the cell surface involves an intermediate low-density compartment, presumably early endosomes.

Structural Analysis of the IGF-II/M6P Receptor in Liver Subcellular Fractions From Control and Insulin-Treated Rats

The structure of the IGF-II/M6P receptor associated with Golgi-endosomal and plasma membrane fractions of untreated and insulin-treated rats was analyzed by reducing SDS-PAGE in combination with affinity cross-linking to IGF-II and Western immunoblotting (Fig. 5). In Golgi-endosomal fractions, a single protein with an apparent molecular weight of about 255 kDa was detected with both methods. In the plasma membrane fraction, however, two distinct proteins were observed, with respective molecular weight of about 255 and 245 kDa. The 255-kDa protein was the main affinity-labeled component, whereas the 245-kDa protein was the major immunoreactive species. Insulin treatment did not affect the size of these proteins but it did modify their abundance in accordance with the changes in IGF-II binding activity. When estimated by scanning densitometry, the abundance of the 255-kDa protein in Golgi-endosomal fractions of insulin-treated rats was decreased by 40–60%, with a maximum at 30 min and a partial reversal at 120 min. Conversely, the abundance of the 245-kDa protein in the plasma membrane fraction was increased by 40–60% from 15 to 120 min.

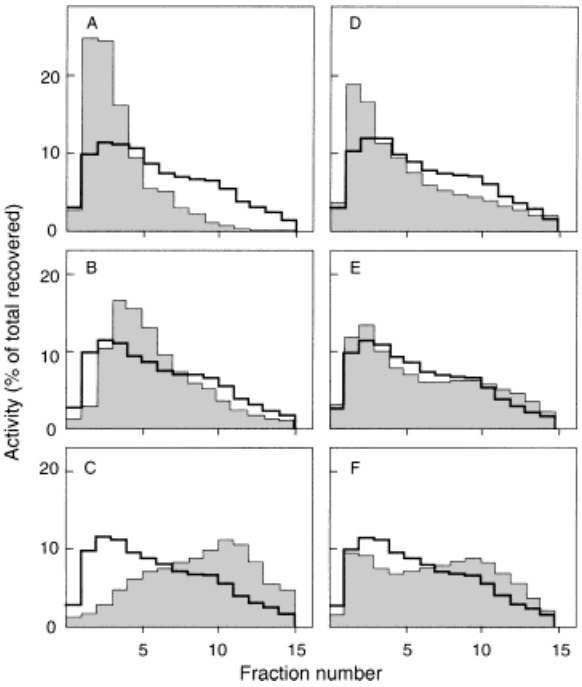

Fig. 4. Distribution of IGF-II binding activity associated with the Golgi-endosomal fraction of control and insulin-treated rats on Percoll density gradients. Combined light and intermediate Golgi-endosomal fractions from control and insulin-treated rats were subfractionated on 7.5-ml Percoll density gradients as described under Materials and Methods. Subfractions (0.5 ml) were assayed for IGF-II binding activity, galactosyltransferase and in vivo uptake of injected ^{125}I-insulin, with results expressed as percentage of total recovered. Left: distribution of IGF-II binding activity in control rats (clear histograms, thick line) compared with the distributions of the following markers (dark histograms, thin line): galactosyltransferase (**A**); ^{125}I-insulin taken up at 90 s (**B**); and ^{125}I-insulin taken up at 8 min (**C**). Right: distribution of IGF-II binding activity in rats killed 15 min (**D**), 30 min (**E**), and 120 min (**F**) after insulin injection (dark histograms, thin line) compared with the distribution of activity in control rats (clear histograms, thick line). The results shown are the mean of four experiments on separate livers.

Effect of Chloroquine Treatment on the Insulin-Induced Changes in IGF-II Receptor Distribution in Liver and IGF-2 Receptor Level in Plasma

Chloroquine has previously been shown to suppress the ability of insulin to induce a redistribution of the IGF-II/M6P receptor to the cell surface in isolated adipocytes [Oka et al., 1987]. To assess whether this occurs in hepatocytes as well, the effect of in vivo chloroquine treatment on the insulin-induced redistribution of the IGF-II/M6P receptor in liver was studied. As shown in Figure 6, chloroquine treatment did not affect IGF-II binding activity in Golgi-endosomal and plasma membrane fractions in the absence of insulin treatment. It did, however, completely suppress the insulin-induced increase in IGF-II binding activity in the plasma membrane fraction and decrease in Golgi-endosomal fractions. Concurrently, chloroquine treatment also suppressed the insulin-induced increase in IGF-II/M6P receptor level in blood plasma as judged on the increase in high-molecular-weight soluble IGF-II binding activity.

DISCUSSION

The ability of insulin to induce a redistribution of intracellular IGF-II receptors to the cell surface was first described in rat adipocytes [Oppenheimer et al., 1983; Oka et al., 1984; Wardzala et al., 1984]. Specifically, insulin was shown to cause an increase in the number of IGF-II receptors at the cell surface and in isolated plasma membrane fractions, and conversely a decrease in receptor number in low density microsomal subfractions; total cellular receptor number was unchanged. These effects were rapid in onset, induced by physiological concentrations of insulin, and fully reversible. In later studies, insulin was found to elicit similar effects in 3T3-L1 adipocytes [Tanner and Lienhardt, 1989], rat skeletal muscle cells [Zhou et al., 1998], H35 rat hepatoma cells [Massague et al., 1982; Corvera et al., 1988], and human skin fibroblasts [Braulke et al., 1989], but, in the two latter cell types, the evidence supporting redistribution of intracellular IGF-II/M6P receptors was indirect. Using cell fractionation techniques, we have extended these observations to normal rat liver cells, presumably hepatocytes that coexpress numerous insulin and IGF-II/M6P receptors. Our results suggest that insulin treatment results in a translocation of the liver IGF-II receptor from Golgi and late endosomes to the plasma membrane via early endosomes.

The subcellular distribution of the liver IGF-II receptor was first assessed using preparative procedures. In control rats, receptor content as judged on IGF-II binding activity was higher in Golgi-endosomal fractions than in the plasma membrane fraction, whether results were related to cell fraction protein (15-fold) or liver weight (30-fold). These results compare favorably to those reported by Scott and Baxter [1990], although perhaps the use of a different method of fractionation led to

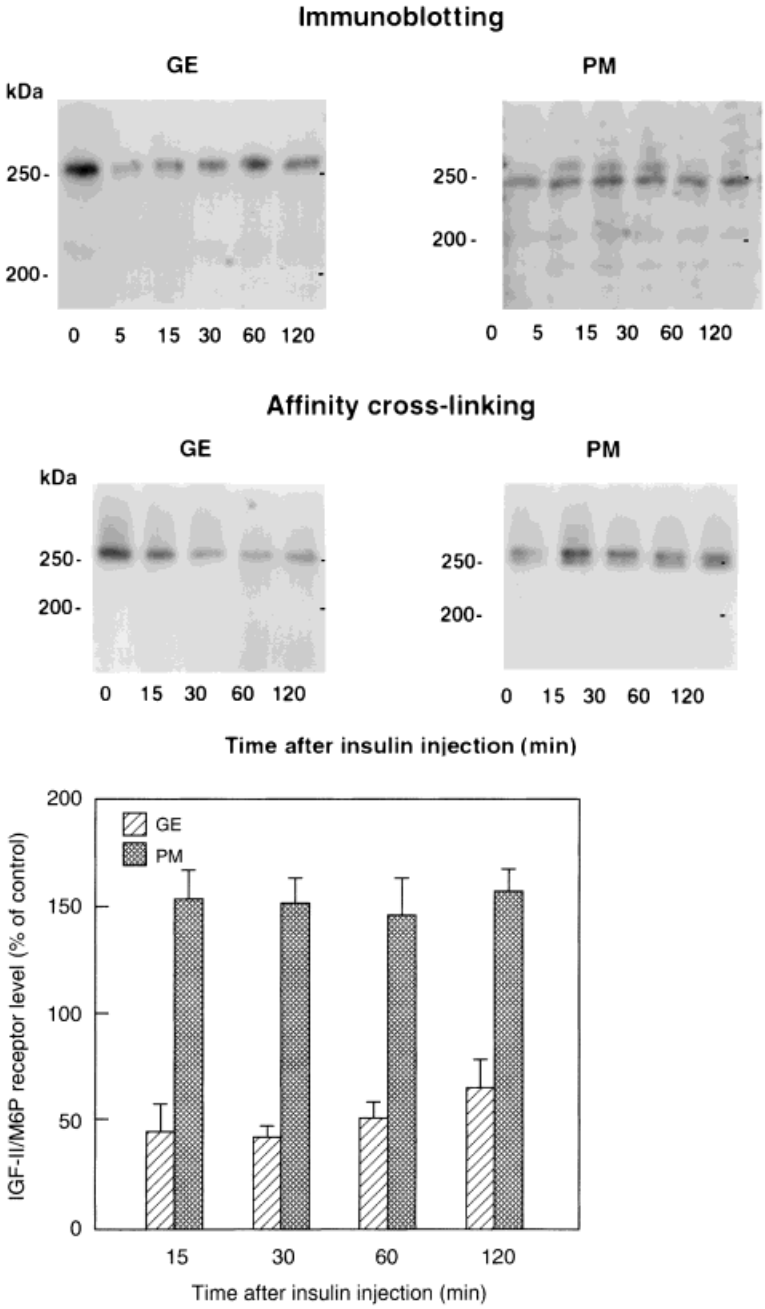

Fig. 5. Affinity cross-linking and Western immunoblotting of the IGF-II/M6P receptor in liver subcellular fractions from control and insulin-treated rats. Combined light and intermediate Golgi-endosomal (GE) fractions and plasma membrane (PM) fractions were prepared from untreated rats and rats killed at the indicated time after insulin injection. They were subjected to SDS-PAGE analysis under reducing conditions, preceded by affinity cross-linking with ^{125}I-IGF-II or followed by Western immunoblotting and detection using ^{125}I-protein A. Top, visualization of affinity-labeled and immune complexes on autoradiography. Bottom, quantitation of the 255 kDa (GE fraction) and 245 (PM fraction) immunoreactive proteins by scanning densitometry. The results shown are the mean ± SEM of three separate determinations.

slightly lower IGF-II binding to our plasma membrane fraction than found by these authors. Insulin treatment led to a decrease in IGF-II binding activity in light and intermediate Golgi-endosomal fractions, along with an increase in binding activity in the plasma membrane fraction; binding activity in the microsomal fraction, which contains 90% of total cellular IGF-II/M6P receptors, was unaffected. These changes in binding activity (1) resulted solely from changes in receptor number; (2) were detectable earlier in the plasma membrane fraction (4 min) than in Golgi-endosomal fractions (15–30 min), with only a partial reversal at 2 h; and (3) exhibited dose dependence closely similar to that of the in vivo insulin-induced changes in the subcellular distribution of the insulin receptor [Desbuquois et al., 1982]. Despite a different experimental design, the redistribution of IGF-II binding activity induced by insulin in liver in vivo compared favorably with that observed in isolated adipo-

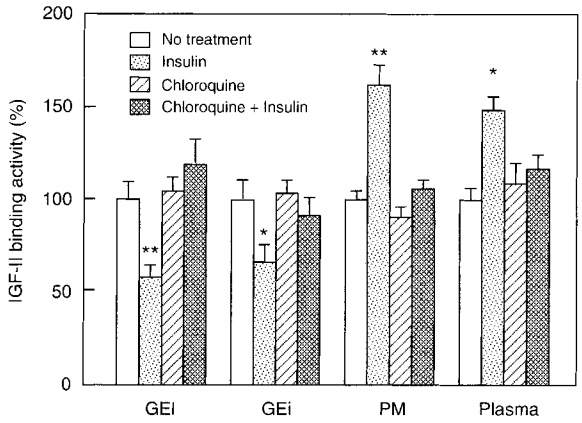

Fig. 6. Effect of chloroquine treatment on IGF-II binding activity of liver cell fractions and plasma IGF-II/M6P receptor level in control and insulin-treated rats. Rats were divided into two groups that were treated or not treated with chloroquine, as described under Materials and Methods. Within each group, some rats received a single injection of insulin (50 μg) at 30 min before killing; other rats were left untreated. Light (GEl) and intermediate (GEi) Golgi-endosomal fractions, plasma membranes (PM), and dense gradient fractions of blood plasma were prepared and assayed for IGF-II binding activity, with results expressed as percentage of activity in untreated rats. The results shown are the mean ± SEM of at least five separate determinations, except those of IGF-II binding activity in plasma of chloroquine-treated rats, which are the mean of three determinations. Asterisks indicate a statistically significant difference from untreated rats (*$P < 0.05$; **$P < 0.01$).

cytes [Oppenheimer et al., 1983; Wardzala et al., 1984]. However, IGF-II binding activity was decreased to a greater extent in liver Golgi-endosomal fractions than in adipocyte low-density microsomal fractions, presumably because the former are more enriched in Golgi and endosomal markers than the latter.

As the plasma membrane fraction originates only in part from the blood sinusoidal domain of the hepatocyte [Neville, 1968] and, together with Golgi-endosomal fractions, accounts for less than 10% of total liver IGF-II binding activity, the microsomal fraction was quantitatively subfractionated on analytical density gradients. In both sucrose and Nycodenz gradients, the distribution of IGF-II binding was closely similar or markedly overlapped with the distributions of galactosyltransferase, a Golgi marker, and of in vivo internalized insulin. It markedly differed, however, from the distribution of 5' nucleotidase and alkaline phosphodiesterase, two plasma membrane markers recovered at higher densities. A comparable distribution of the liver bovine IGF-II/M6P receptor as assessed by Western immunoblotting has previously been reported by Messner et al. [1989]. Insulin treatment led to a slight, albeit reproducible shift of IGF-II binding activity toward higher densities on sucrose gradients, suggesting a translocation of intracellular IGF-II/M6P receptors to the plasma membrane. Based on the magnitude of this shift, the percentage of IGF-II binding activity translocated was about 12% at 4 min and 8% at 15 min. In time studies, the insulin-induced density shift in IGF-II binding activity did not exactly match the changes in binding activity in plasma membrane and Golgi-endosomal fractions, probably reflecting differences in the preparative and analytical methods of fractionation.

Previous studies using ^{125}I-insulin as an endocytic marker have shown that, upon subfractionation of Golgi-endosomal fractions on Percoll density gradients, Golgi elements and early endosomes are recovered at low densities in the upper region of the gradient, whereas late endosomes migrate at higher densities in the lower region [Khan et al., 1982]. The present studies confirm these findings and show that, in the basal state, IGF-II binding activity is broadly distributed throughout the gradient, with about 55% in the upper, low density region. Insulin treatment led to a shift of binding activity toward the upper region of the gradient, suggesting a translocation of intracellular IGF-II/M6P receptors from late endosomes to Golgi or early endosomes, more likely the latter. Later, however, this pattern was reversed, and IGF-II binding activity was shifted toward the lower region of the gradient, suggesting an association of receptors internalized from the cell surface with late endosomes. Because of the similar distributions of Golgi and early endosomes, the relative proportions of the IGF-II receptors present in these organelles could not be determined in either control or insulin-treated rats. Based on the relative enrichments of galactosyltransferase and IGF-II/M6P receptors in membranes immunoadsorbed to a specific antibody conjugated to polystyrene beads, Messner et al. [1989] have estimated that at least 70% of the receptors in a bovine Golgi-endosomal fraction are associated with endosomes.

It has been previously shown that, in addition to decreasing protein recovery in Golgi-endosomal fractions, in vivo insulin treatment causes slight changes in the specific activity of

5' nucleotidase and alkaline phosphodiesterase in these fractions, as well as in the plasma membrane fraction [Desbuquois et al., 1982]. Although somewhat ressembling the changes in IGF-II binding activity described here, these changes were of lesser magnitude and more delayed. These findings suggest that the insulin-induced redistribution of the IGF-II receptor is relatively selective. However, insulin may also causes the redistribution of other receptors present in liver. Likely candidates are the transferrin receptor, which has been shown to undergo insulin-induced redistribution in adipocytes [Davis et al., 1986], 3T3-L1 adipocytes [Tanner and Lienhardt, 1987, 1989], and skeletal muscle cells [Zhou et al., 1998], as well as the α_2-macroglobulin receptor, which is also insulin sensitive in 3T3L1 cells [Corvera et al., 1989]. Furthermore, immunochemical and immunocytochemical evidence has been presented that the IGF-II/M6P and transferrin receptors are in part colocalized and use the same membrane compartments as Glut 4 for insulin-dependent trafficking [Kandror and Pilch, 1996].

Chloroquine, a weak base that disrupts the acidification of endomembrane compartments and interferes with the recycling of many membrane receptors, has previously been shown to inhibit the insulin-induced redistribution of the IGF-II/M6P receptor in isolated adipocytes [Oka et al., 1987]. In agreement with these observations, our studies show that, when administered in vivo under conditions known to cause accumulation of undegraded insulin and insulin receptors in endosomes [Posner et al., 1982; Desbuquois et al., 1992], chloroquine fully inhibits the insulin-induced changes in IGF-II binding activity in plasma membranes and Golgi-endosomal fractions. These findings suggest that acidification of the exocytic pathway may be required for insulin-induced redistribution of the IGF-II/M6P receptor. However, other mechanisms may be involved as well, since chloroquine has been shown to inhibit the insulin-induced redistribution of Glut 4 in adipocytes independent of its action on endomembrane pH [Romanek et al., 1993].

Reducing SDS-PAGE analysis of the IGF-II receptor associated with Golgi-endosomal fractions showed a single protein of about 250–260 kDa, whether affinity cross-linking or Western immunoblotting was used. This molecular weight is in agreement with previous estimates of 250–260 kDa under reducing conditions and 210–220 kDa under nonreducing conditions [Massague and Czech, 1982; August et al., 1983; Oppenheimer and Czech, 1983; Scott and Baxter, 1987, 1990]. However, the IGF-II receptor associated with the plasma membrane fraction migrated under reducing conditions, as two proteins, of molecular size 245 and 255 kDa, respectively; the 245-kDa protein was the major immunoreactive protein, while the 255-kDa protein was the major affinity-labeled species. Presumably, the 245-kDa protein was generated by the removal of a short portion of the extracellular and/or cytoplasmic domains of the native receptor. This could reflect either the higher intrinsic proteolytic activity of plasma membranes relative to Golgi and endosomes, or the artifactual release of lysosomal proteases during liver homogenization in hypotonic medium. In support of the latter possibility, the IGF-II receptor associated with plasma membranes isolated from liver homogenates in isotonic sucrose appeared to exhibit, on Western immunoblotting, the same apparent molecular size as that associated with Golgi fractions [Scott and Baxter, 1990].

Baxter and coworkers previously showed that the changes in the content of hepatic IGF-II receptor induced by development and liver regeneration are accompanied by parallel changes in the level of serum soluble receptor [Bobek et al., 1992; Scott and Baxter, 1996]. This finding has led to the suggestion that—at least in adult rats—liver is the major source of soluble receptor. Our observations in time studies demonstrating that the insulin-induced redistribution of the liver IGF-II receptor is accompanied by an increase in the level of soluble receptors in blood plasma, and that both events are suppressed by chloroquine treatment, support this view. Whether proteolytic solubilization of the liver IGF-II receptor under physiological conditions occurs at the plasma membrane or intracellularly remains unclear. In the liver cell line BRL, the release of soluble receptor was inhibited by serine protease inhibitors, suggesting extracellular proteolysis [Clairmont and Czech, 1991]. By contrast, in primary cultures of rat hepatocytes, soluble receptor release was increased by microtubule disrupting agents, suggesting endosomal proteolysis [Scott and Baxter, 1996]. Additional studies are required to determine the subcellular site of IGF-II/M6P receptor prote-

olysis in liver and identify the proteases involved in this process.

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