

# Localization of Transferrin Receptors and Insulin-like Growth Factor II Receptors in Vesicles from 3T3-L1 Adipocytes That Contain Intracellular Glucose Transporters

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**Abstract.** Transferrin receptors in detergent extracts of subcellular membrane fractions prepared from 3T3-L1 adipocytes were measured by a binding assay. There was a small but significant increase (1.2-fold) in the amount of receptor in a crude plasma membrane fraction and a 40% decrease in the number of transferrin receptors in microsomal membranes prepared from insulin-treated cells, when compared with corresponding fractions from control cells. Intracellular vesicles containing insulin-responsive glucose transporters (GT) have been isolated by immunoadsorption from the microsomal fraction (Biber, J. W., and G. E. Lienhard. 1986. *J. Biol. Chem.* 261:16180–16184). All of the transferrin receptors in this fraction were localized in these vesicles; however, because the GT vesicles contain ~30-fold fewer transferrin receptors than GT, on the average only one vesicle in three contains a transferrin receptor.

The binding of <sup>125</sup>I-pentamannose 6-phosphate BSA

to 3T3-L1 adipocytes at 4°C was used to monitor surface insulin-like growth factor II (IGF-II)/mannose 6-phosphate receptors. Exposure of cells to insulin at 37°C for 5 min resulted in a 2.5–4.5-fold increase in surface receptors. There was a corresponding 20% decrease in the amount of IGF-II receptors in the microsomal membranes prepared from insulin-treated cells, as assayed by immunoblotting. Moreover, the IGF-II receptors and GT were located in the same intracellular vesicles, since antibodies to the carboxy-terminal peptide of either protein immunoadsorbed vesicles containing 70–95% of both proteins initially present in the microsomal fraction. In conjunction with other studies, these results indicate that in 3T3-L1 adipocytes, three membrane proteins (the GT, the transferrin receptor, and the IGF-II receptor) respond similarly to insulin, by redistributing to the surface from intracellular compartment(s) in which they are colocalized.

**T**HE rate of glucose transport into rat adipocytes is increased in response to insulin. At the subcellular level, insulin elicits a decrease in the number of intracellular glucose transporters (GT)<sup>1</sup>, which is accompanied by a corresponding increase in the number of plasma membrane GT<sup>1</sup> (Cushman and Wardzala, 1980; Suzuki and Kono, 1980). The translocation of GT to the plasma membrane contributes to the insulin-stimulated increase in the rate of glucose transport. A similar mechanism of insulin action has been described in mouse 3T3-L1 adipocytes (Biber and Lienhard, 1986; Blok et al., 1988). Vesicles containing the insulin-responsive intracellular GT have recently been isolated from 3T3-L1 adipocytes (Biber and Lienhard, 1986) and from rat adipocytes (James et al., 1987).

It is known that insulin also elicits translocation to the plasma membrane of the transferrin receptor in rat and in 3T3-L1 adipocytes (Davis et al., 1986; Tanner and Lienhard,

1987), and of the insulin-like growth factor II (IGF-II) receptor<sup>2</sup> in rat adipocytes (Oka et al., 1984; Wardzala et al., 1984). In this study, we have examined the effect of insulin on the distribution of the transferrin receptor and the IGF-II receptor between a subcellular fraction from 3T3-L1 adipocytes containing the plasma membranes and one containing almost exclusively intracellular membranes. Moreover, the content of these receptors in the vesicles containing the insulin-responsive intracellular GT has been examined. The results provide further evidence that these two receptors are translocated in response to insulin, and demonstrate that the insulin-responsive pools of GT, transferrin receptor, and

2. Although it has been shown that the IGF-II receptor is identical with the cation-independent mannose 6-phosphate receptor, this protein will simply be referred to as the IGF-II receptor. Antibodies that have been raised against this protein will be referred to as mannose 6-phosphate receptor antibodies when the antigen was purified by affinity chromatography on phosphomannan-Sepharose (gift of D. Messner and S. Kornfeld, Washington University School of Medicine, St. Louis, MO), or as IGF-II receptor antibodies when the antigen was purified by affinity chromatography on IGF-II coupled to Affi-gel 15 (gift of C. Scott and R. Baxter, Royal Prince Alfred Hospital, Camperdown, Australia).

1. *Abbreviations used in this paper:* C<sub>12</sub>E<sub>8</sub>, octaethylene glycol dodecyl ether; GT, glucose transporter; IGF-II, insulin-like growth factor II; KRP, Krebs-Ringer phosphate buffer; PMP-BSA, pentamannose 6-phosphate BSA.

IGF-II receptor reside largely in the same intracellular membranes.

## Materials and Methods

### Antibodies

Affinity-purified rabbit antibodies against the purified human erythrocyte GT (Schroer et al., 1986) were used for detection of GT on immunoblots. Affinity-purified rabbit antibodies against the carboxy-terminal peptide (residues 477-492) of the human/rat GT (GT C-peptide antibodies) (Davies et al., 1987) were used for isolation of intracellular GT vesicles. Whole rabbit antiserum against the rat liver IGF-II receptor (kindly donated by C. Scott and R. Baxter, Royal Prince Alfred Hospital, Camperdown, Australia; Scott and Baxter, 1987) and against the bovine cation-independent mannose 6-phosphate receptor (a gift from D. Messner and S. Kornfeld, Washington University School of Medicine, St. Louis, MO) were used for detection of the IGF-II receptor on immunoblots. Whole rabbit antiserum against the carboxy-terminal peptide of the bovine mannose 6-phosphate receptor (also kindly donated by D. Messner and S. Kornfeld) was used for immunoprecipitation of the IGF-II receptor from solubilized GT vesicles (Fig. 4).

We prepared affinity-purified rabbit antibodies against the carboxy-terminal peptide (C-peptide) of the rat placental IGF-II receptor (MacDonald et al., 1988). A peptide consisting of residues 2,048-2,060 (with cys-gly added to the NH<sub>2</sub>-terminus) was synthesized on a Bioscience (San Rafael, CA) 9500 solid phase peptide synthesizer and purified by T. Ciardelli, Dartmouth Medical School (Ciardelli et al., 1988). The peptide was coupled to keyhole limpet hemocyanin using *m*-maleimidobenzoyl-*N*-hydroxysulfosuccinimide ester (Pierce Chemical Co., Rockford, IL). New Zealand rabbits were immunized at multiple intradermal sites with 60 µg peptide coupled to 600 µg keyhole limpet hemocyanin in complete Freund's adjuvant, and boosted 4 wk later with 30 µg peptide coupled in incomplete Freund's adjuvant. Antibodies were purified from 4 ml serum (collected 2 mo after the time of the first immunization) by affinity chromatography at 4°C, using a column (2 ml) of IGF-II receptor peptide (7 mg) coupled to Affi-gel 15 (Bio-Rad Laboratories, Richmond, CA). After application of the serum, the column was washed with 20 ml PBS, and the bound antibodies were eluted with 0.2 M glycine, pH 2.5. The pH of the collected fractions was subsequently adjusted to 7.4 using 2 M Tris. The protein content of the fractions was determined (Peterson, 1977), and immunoreactivity in the pooled fractions (2.25 mg protein) was assessed by an ELISA against the IGF-II receptor C-peptide.

### Cell Culture

3T3-L1 fibroblasts were cultured and differentiated as previously described (Frost and Lane, 1985). Mature 3T3-L1 adipocytes were used between 8 and 12 d after initiation of differentiation, at which time >95% of the cells exhibited the adipocyte phenotype. Cells were incubated for 2 h in serum-free Dulbecco's modified Eagle's medium (Gibco Laboratories, Grand Island, NY) at the beginning of each experiment.

### Cell Fractionation

A low-density microsomal fraction was prepared from 3T3-L1 adipocytes by a modification (Brown et al., 1988) of a previously published procedure (Biber and Lienhard, 1986). 3T3-L1 adipocytes (6-cm dishes; ~4 × 10<sup>6</sup> cells/dish) were treated without or with porcine insulin (100 nM, final; a gift from Eli Lilly, Indianapolis, IN or purchased from Sigma Chemical Co., St. Louis, MO) and incubated at 37°C in 10% CO<sub>2</sub> for the desired time. The plates were then transferred to ice, and the cell monolayers were washed twice with cold KCl buffer (150 mM KCl, 20 mM Hepes, 2 mM MgSO<sub>4</sub>, pH 7.4 at 4°C). The cells were scraped from each plate with a rubber policeman in 1.7 ml of cold KCl buffer containing protease inhibitors (200 µM phenylmethylsulfonyl fluoride, 1.5 µM pepstatin A, and 10 µM *L*-trans-epoxysuccinylleucylamido-[3-methyl]butane) and transferred to a 30-ml homogenizer tube on ice (A. H. Thomas Scientific, Philadelphia, PA, 3431-D88). The plate was rinsed with an additional 1.7 ml homogenization buffer; this wash was also transferred to the homogenizer tube, and cells were homogenized on ice with 25 hand strokes with a Teflon pestle. The homogenate was centrifuged at 16,000 *g*<sub>max</sub> for 20 min at 4°C, followed by careful aspiration of the fat layer. The pellet contains 95% of the plasma membrane (Brown et al., 1988) and will be referred to as the crude plasma membrane fraction. The supernatant, designated the microsomal fraction, contains 50% of the total cellular GT. The GT in this fraction are insulin-

responsive, since there is typically half as much GT in the microsomal fraction prepared from insulin-treated cells as from basal cells (see Fig. 3). There is a corresponding increase in the GT content of the crude plasma membrane fraction, from 50 (basal cells) to 75% (insulin-treated cells) of the total GT (Brown et al., 1988). Intracellular vesicles were immunoadsorbed from the microsomal fraction (see below) or the membranes in the microsomal fraction were sedimented by ultracentrifugation at 180,000 *g*<sub>max</sub> for 1 h at 4°C. When 3T3-L1 adipocytes were harvested from 10-cm dishes (10<sup>7</sup> cells/plate), the same fractionation procedure was carried out except that the cells were homogenized in a total volume of 8 ml.

### Immunoabsorption of the GT-containing Vesicles and IGF-II Receptor-containing Vesicles

GT-containing vesicles were immunoabsorbed by a previously published procedure (Biber and Lienhard, 1986). Formaldehyde-fixed *Staphylococcus aureus* cells (Pansorbin; Calbiochem-Behring Corp., La Jolla, CA) were extracted with SDS-mercaptoethanol (Richert et al., 1979) and stored at -70°C. The *S. aureus* cells were washed twice with KCl buffer containing 1% BSA, and then incubated with GT C-peptide antibodies for 2 h at room temperature on a rotating wheel. The antibody-coated *S. aureus* cells were then washed twice with KCl buffer. An aliquot of the microsomal fraction (1 ml, equivalent to 12.5% of a 10-cm plate) was incubated with antibody-coated *S. aureus* cells for 2 h at room temperature on a rotating wheel. The *S. aureus* cells with the adsorbed GT vesicles were then pelleted in an Eppendorf microfuge (Brinkman Instruments Co., Westbury, NY) and the supernatant was saved. The protein in the immunoabsorbed supernatant (1 ml) was precipitated with TCA (10% wt/vol) and resuspended in SDS sample buffer.

A similar procedure was used to isolate intracellular vesicles containing IGF-II receptors, with the modification that aliquots of the microsomal fraction were incubated with *S. aureus* cells coated with the affinity-purified antibodies against the C-peptide of the rat IGF-II receptor. The *S. aureus* cells with adsorbed IGF-II receptor vesicles were then pelleted, and the supernatant was saved and prepared for SDS-PAGE as described above.

### Binding Assay for Solubilized Transferrin Receptor

Vesicles immunoabsorbed to *S. aureus* cells or membrane pellets were resuspended in KCl buffer containing 1% Triton X-100, 1 mg/ml BSA, and protease inhibitors (as above). Transferrin<sup>3</sup> binding to soluble receptors was measured by a modification of previously published procedures (Ciechanover et al., 1983; Lamb et al., 1983). Samples (100 µl; typically containing membranes or vesicles derived from 500,000 cells) were incubated with 100 µl of <sup>125</sup>I-transferrin, which was prepared as previously described (Tanner and Lienhard, 1987) (final concentration, 0.3 nM; 40,000 cpm/assay) in KCl buffer containing 1% BSA. After 1-2 h incubation at 4°C (by which time the binding was at equilibrium), 100 µl of 2 mg/ml human gamma-globulin (Sigma Chemical Co.) and 245 µl of cold, saturated (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> (final concentration, 45% saturation) were added. The samples were kept on ice for 10 min, and, after the addition of 1 ml 45% (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> in cold KCl buffer, were filtered through 24-mm Whatman Inc. (Clifton, NJ) GFC glass-fiber filters. The filters were washed twice with 1-ml aliquots of 45% (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> in cold KCl buffer, dried, and radioactivity was determined in a Beckman Instruments, Inc. (Fullerton, CA) gamma counter. A control experiment, in which the period of incubation in ammonium sulfate was shortened from 10 to 5 min, showed that there was no dissociation of the precipitated transferrin from its receptor during the 10 min. All results have been corrected for nonspecific binding, which was taken as the amount of <sup>125</sup>I-transferrin bound in the presence of excess (1 µM) unlabeled transferrin. The amount of nonspecific binding was independent of the presence of added cell extract, and was thus due to ammonium sulphate precipitation of free <sup>125</sup>I-transferrin.

In a control experiment where specific binding of <sup>125</sup>I-transferrin to soluble receptors was measured after incubation with varying concentrations of ammonium sulfate (20-55% saturation), similar levels of specifically bound <sup>125</sup>I-transferrin were measured at 45, 50, and 55% ammonium sulfate, but the level of nonspecific binding increased with increasing amounts of ammonium sulfate. This result suggests that the soluble transferrin receptors are entirely precipitated by 45% ammonium sulfate.

The dissociation rate of <sup>125</sup>I-transferrin from its receptor was measured by incubation of aliquots of a Triton X-100 (1%) cell extract with <sup>125</sup>I-transferrin (1 nM) at 4°C for 50 min, followed by the addition of unlabeled transferrin (1 µM) and measurement of the amount of specifically bound transferrin remaining after various times at 4°C. The first-order rate con-

3. Diferric transferrin will be referred to as transferrin for the sake of brevity.

stant for dissociation ( $k_{-1}$ ) was  $0.038 \text{ min}^{-1}$ . The rate of association of transferrin with its receptor was measured by incubation of a Triton X-100 (1%) cell extract with  $3 \text{ nM}$   $^{125}\text{I}$ -transferrin at  $4^\circ\text{C}$  for 15 s–50 min, followed by measurement of specific binding. Equilibrium binding was achieved within 7 min, and the half-time was  $\sim 2 \text{ min}$ . The rate constant for association ( $k_{+1}$ ) was calculated to be  $1.1 \times 10^8 \text{ min}^{-1}\text{M}^{-1}$ . The kinetically derived value ( $k_{-1}/k_{+1}$ ) for the dissociation constant,  $K_d$ , is thus  $0.35 \text{ nM}$ , which agrees well with the value derived from equilibrium binding of  $^{125}\text{I}$ -transferrin ( $0.5 \text{ nM}$ , see Fig. 1).

### Preparation of $^{125}\text{I}$ -Pentamannose 6-Phosphate-BSA (PMP-BSA)

Pentamannose 6-phosphate (160 mg; a gift from M. E. Slodki, United States Department of Agriculture, Peoria, IL) and BSA (12 mg; Sigma Chemical Co., A-7638) in 1 ml  $50 \text{ mM}$  *N,N*-bis(2-hydroxyethyl)glycine, pH 9.0, at  $37^\circ\text{C}$ , were incubated with sodium cyanoborohydride (9.8 mg; Aldrich Chemical Co., Milwaukee, WI) for 45 h at  $37^\circ\text{C}$  (Braulke et al., 1987). The reaction mixture containing PMP-BSA was then dialyzed 24 h at room temperature against 1 liter of PBS, with one buffer change. Coupling of PMP-BSA resulted in an increase in its apparent  $M_r$  from 59,000 to 76,000, as determined by SDS-PAGE under nonreducing conditions.

PMP-BSA ( $100 \mu\text{g}$  in  $100 \mu\text{l}$  PBS) was incubated with  $\text{Na-}^{125}\text{I}$  (1 mCi; Amersham Corp., Arlington Heights, IL) in a glass test tube coated with  $10 \mu\text{g}$  iodogen (Pierce Chemical Co.) for 15 min at room temperature.  $^{125}\text{I}$ -PMP-BSA (sp act  $9.6 \text{ Ci/g}$ ) was isolated by gel filtration (Sephadex G-25) in PBS, made  $1 \text{ mg/ml}$  in BSA, and stored at  $-70^\circ\text{C}$ .

### $^{125}\text{I}$ -PMP-BSA Binding to Cells at $4^\circ\text{C}$

Cell monolayers in a six-well cluster dish ( $35 \text{ mm/well}$ ;  $2 \times 10^6$  cells/well) were washed twice with 1-ml aliquots of Krebs-Ringer phosphate buffer (KRP;  $128 \text{ mM}$  NaCl,  $4.7 \text{ mM}$  KCl,  $1.25 \text{ mM}$   $\text{MgSO}_4$ ,  $1.25 \text{ mM}$   $\text{CaCl}_2$ ,  $5 \text{ mM}$   $\text{NaH}_2\text{PO}_4$ , pH 7.4) at  $37^\circ\text{C}$ , followed by the addition of  $995 \mu\text{l}$  of KRP and, when desired,  $5 \mu\text{l}$  insulin ( $100 \text{ nM}$ , final). At the appropriate time, buffer was removed from all wells of a six-well plate, and cell monolayers were washed rapidly three times with ice-cold PBS. The six-well plate was then transferred to ice and 1 ml of cold KRP was added to each well. Subsequently, buffer was replaced with 1 ml  $^{125}\text{I}$ -PMP-BSA ( $1 \text{ nM}$ ;  $100,000 \text{ cpm/well}$ ) in cold KRP containing  $1 \text{ mg/ml}$  BSA and cells were incubated at  $4^\circ\text{C}$ . At the end of 3 h (at which time binding was at equilibrium), unbound ligand was aspirated, and nonspecific binding was reduced by three 1-ml (1 min each) washes with cold KRP. Cells were solubilized with 2 ml of 1 N NaOH, and the radioactivity was determined in a Beckman Instruments, Inc. gamma counter. A control experiment, in which the efficacy of three rapid washes was compared with three 1-min washes, indicated that none of the specifically bound  $^{125}\text{I}$ -PMP-BSA was lost in the latter procedure, whereas the nonspecific binding was reduced. Nonspecific binding was taken as the  $^{125}\text{I}$ -PMP-BSA bound in the presence of  $5 \text{ mM}$  mannose 6-phosphate (Sigma Chemical Co.); the same level of nonspecific binding was observed in the presence of  $1 \mu\text{M}$  PMP-BSA.  $^{125}\text{I}$ -PMP-BSA binds specifically to the IGF-II receptor, since the presence of  $5 \text{ mM}$  mannose 1-phosphate (Sigma Chemical Co.) does not reduce the amount of bound ligand. In addition, the omission of  $\text{CaCl}_2$  and  $\text{MgSO}_4$  from the binding buffer or the addition of EDTA ( $5 \text{ mM}$ ) had no effect on the amount of  $^{125}\text{I}$ -PMP-BSA bound to basal and insulin-treated cells.

### Gel Electrophoresis and Immunoblotting

Final concentrations in SDS sample buffer were 4% SDS (Pierce Chemical Co.; Lauryl brand), 10% (vol/vol) glycerol,  $1 \text{ mM}$  EDTA,  $95 \text{ mM}$  Tris-HCl (pH 6.8),  $120 \mu\text{g/ml}$  bromphenol blue, and protease inhibitors ( $1 \text{ mM}$  diisopropyl fluorophosphate,  $10 \mu\text{M}$  *L-trans*-epoxysuccinylleucylamido-[4-methyl]butane, and  $1.5 \mu\text{M}$  pepstatin A). SDS-PAGE was carried out as previously described (Gibbs et al., 1986) under nonreducing conditions, using 5 and 7.5% (wt/vol) acrylamide, respectively, for the stacking and separating gels. For immunoblotting, samples were electrophoretically transferred to  $0.2 \mu\text{m}$  nitrocellulose (BA83; Schleicher & Schuell, Inc., Keene, NH) in  $25 \text{ mM}$  sodium phosphate (pH 6.5) for 2 h at  $350 \text{ mA}$ . The blots were then blocked with BSA; treated with affinity-purified GT antibodies, and/or with antiserum against the rat liver IGF-II receptor, or bovine mannose 6-phosphate receptor (see figure legends for details); and then labeled with  $^{125}\text{I}$ -labeled goat antibodies against rabbit IgG (New England Nuclear, Boston, MA) at  $1.2 \times 10^5 \text{ dpm/ml}$  as described elsewhere (Lienhard et al., 1982).

For quantitation of the immunoblots, autoradiograms were aligned over

the nitrocellulose, and labeled bands were cut out and counted in a gamma counter. Each value was corrected for background by cutting and counting a region within the same lane that exhibited no labeled band. It was shown by the application of SDS samples containing various amounts of 3T3-L1 membranes that the amount of  $^{125}\text{I}$ -labeled goat antibodies against rabbit IgG specifically bound to the GT and IGF-II receptor was proportional to the amount of protein applied over the range used for quantitation in this study.

## Results

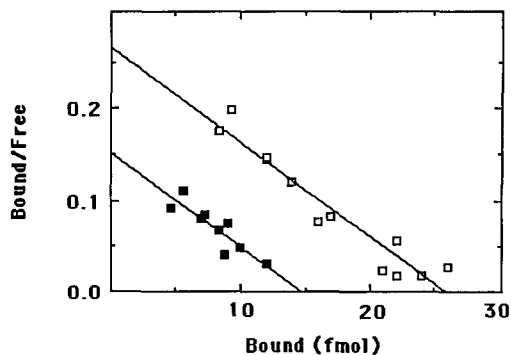
### Effect of Insulin on the Subcellular Distribution of Transferrin Receptors

Insulin elicits a redistribution of transferrin receptors in both rat adipocytes (Davis et al., 1986) and in 3T3-L1 adipocytes (Tanner and Lienhard, 1987). After a 5-min exposure to insulin at  $37^\circ\text{C}$ , there is a twofold increase in surface receptors on 3T3-L1 adipocytes. There is a corresponding decrease in intracellular receptors, as assayed by the decrease in intracellular  $^{125}\text{I}$ -transferrin at steady state (Tanner and Lienhard, 1987). Further evidence for insulin-stimulated translocation of transferrin receptors has been obtained through measurement of the transferrin receptor content of subcellular fractions prepared from basal and insulin-treated cells (Table I). The amount of transferrin bound to receptors in the crude plasma membrane fraction increased from 183 to 238 fmol per 10-cm plate in response to insulin, as measured by binding of  $^{125}\text{I}$ -transferrin at a single concentration (Table I). In six experiments of this type, the fold increase in the amount of transferrin bound to receptors in the crude plasma membrane fraction averaged  $1.2 \pm 0.04$  (SEM). Although at first glance this value may appear to be in disagreement with the twofold increase in surface receptors observed in intact cells, the data are in fact consistent. The reason is that the crude plasma membrane fraction contains intracellular as well as surface transferrin receptors. This fraction contained  $71 \pm 2\%$  (SEM,  $n = 6$ ) of the total receptors in the basal state and  $83 \pm 1\%$  (SEM,  $n = 6$ ) in the insulin-treated state. On the other hand, only 15% of the total receptors in the cell are on the surface in the basal state, as assayed by the steady-state distribution of  $^{125}\text{I}$ -transferrin (Tanner and Lienhard, 1987). Thus, the increase of receptors in the crude plasma membrane fraction by 12% of the total is consistent with the

Table I. Subcellular Localization of Transferrin Receptors

Condition	$^{125}\text{I}$ -Transferrin bound (fmol/fraction per 10-cm dish)		
	Crude plasma membranes	Microsomal membranes	GT vesicles
Basal	$183 \pm 10$	$96 \pm 9$	$107 \pm 13$
Insulin	$238 \pm 9$	$58 \pm 7$	$62 \pm 3$

Cells were treated with or without insulin ( $100 \text{ nM}$ , final) for 5 min at  $37^\circ\text{C}$ . Cells were then homogenized and fractionated as described in Materials and Methods, and transferrin binding to receptors in the solubilized membrane fractions and GT vesicles was assayed as described in Materials and Methods. GT vesicles were isolated by adsorbing 1 ml of the microsomal fraction with  $2 \mu\text{l}$  of *S. aureus* cells coated with  $3 \mu\text{g}$  of GT C-peptide antibodies. Transferrin bound to vesicles adsorbed to nonimmune IgG-coated *S. aureus* cells was routinely 20–35% of the amount of transferrin bound to vesicles adsorbed to GT antibody-coated *S. aureus* cells (data not shown). The results are presented as the mean  $\pm$  SD of duplicate determinations of fractions prepared from duplicate basal and insulin-treated plates. Similar results were obtained in a separate, independent experiment. In addition, separate experiments in which the receptors in the crude plasma membranes and the microsomal membranes ( $n = 2$ ) or in the crude plasma membranes and the GT vesicles ( $n = 2$ ) were assayed in this way yielded similar results.



**Figure 1.** Effect of insulin treatment on transferrin receptor content in the microsomal membranes. Cells were treated without (□) or with (■) insulin (100 nM, final) for 5 min at 37°C, followed by transfer of cell monolayers to ice, and isolation of the microsomal membranes, as described in Materials and Methods. Transferrin binding to the solubilized receptors was then measured as described in Materials and Methods, with a fixed amount of <sup>125</sup>I-transferrin and increasing concentrations of unlabeled transferrin. 10% of the microsomal membranes from a 10-cm plate was used per assay. The points shown are the individual values from duplicate determinations at each ligand concentration, and are representative of results obtained in four independent experiments.

amount necessary for the surface receptors to increase by twofold (from 15 to 30% of the total).

The supernatant from the 16,000 g centrifugation contains microsomal membranes, which can be pelleted upon centrifugation at 180,000 g (see Materials and Methods). Transferrin binding to receptors in the microsomal membranes prepared from basal and insulin-treated cells was also measured. Insulin elicited a reduction in the amount of transferrin bound, from 96 to 58 fmol/10-cm plate (Table I). This result was representative of four separate experiments, in which there was a  $41 \pm 4\%$  (SEM) decrease in the amount of transferrin bound to receptors in the microsomal membranes from insulin-treated cells. There was no change in total cellular transferrin receptor binding (crude plasma membranes plus microsomal membranes) in response to insulin ( $n = 4$ ). These results further support the conclusion that insulin elicits translocation of transferrin receptors in 3T3-L1 adipocytes.

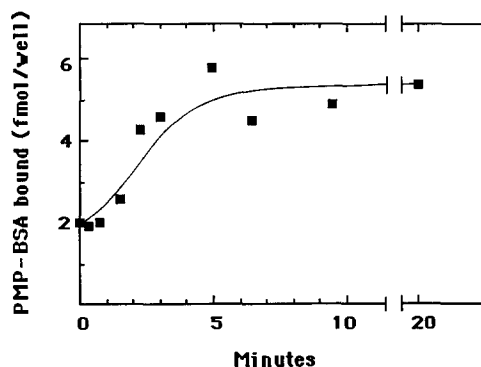
In principle, the changes in the amount of transferrin bound to receptors in the crude plasma membrane and microsomal membranes prepared from insulin-treated cells (Table I) could result from changes in receptor affinity rather than changes in receptor number. To check that this was not the case, Scatchard analysis of transferrin binding to receptors in the microsomal membranes prepared from basal and insulin-treated cells was performed, and results from a representative experiment are shown in Fig. 1. In four independent experiments, there was a 45% reduction in the amount of transferrin receptors from  $273 \pm 12$  (SEM) to  $150 \pm 3$  fmol/10-cm dish in response to insulin. There was no change in receptor affinity; the values for the dissociation constant were  $0.56 \pm 0.04$  (SEM) and  $0.51 \pm 0.02$  nM for the receptors in the microsomal membranes prepared from basal and insulin-treated cells, respectively.

Insulin also elicits translocation of GT in 3T3-L1 adipocytes. When subcellular fractions are prepared from basal cells and from cells exposed to 100 nM insulin for 15 min

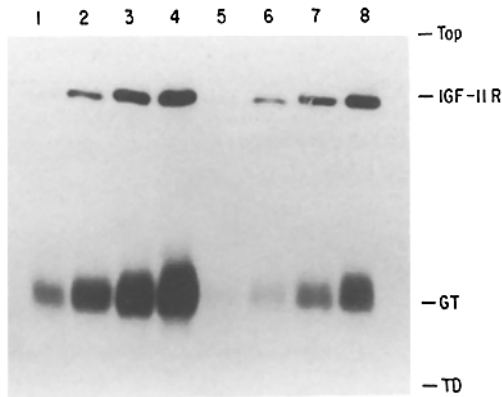
at 37°C, there is a 1.5-fold increase in GT content in the crude plasma membrane fraction (from 50 to 75% of the total GT), and a 50% decrease in GT content in the microsomal fraction of insulin-treated cells (from 50 to 25% of the total GT) compared with the corresponding fractions prepared from basal cells (Brown et al., 1988). The microsomal fraction prepared from cells exposed to 100 nM insulin for 5 min, instead of 15 min, at 37°C (conditions which were used in this paper [Table I and Fig. 1] and which are optimal for translocation of transferrin receptor [Tanner and Lienhard, 1987]) exhibited a  $39 \pm 3\%$  ( $n = 6$ ; SEM) decrease in GT content when compared with the microsomal fraction from basal cells (data not shown). The GT content of the crude plasma membrane fraction under these conditions was not examined. Thus, the insulin-induced change in the subcellular distribution of transferrin receptors is paralleled by a similar change in the subcellular distribution of GT.

### Localization of Transferrin Receptors in the Intracellular GT Vesicles

Our laboratory has optimized a procedure for isolation of vesicles containing insulin-responsive intracellular GT (Biber and Lienhard, 1986; Brown et al., 1988). Since insulin elicits translocation of the GT and the transferrin receptor, we next examined whether transferrin receptors are located in the intracellular GT vesicles. As shown in Table I, there were equivalent amounts of transferrin bound to microsomal membranes and to GT vesicles isolated from parallel aliquots of the microsomal fraction, from both basal and insulin-treated cells. Thus, all of the transferrin receptors in the microsomal fraction were contained in the GT vesicles. There were 107 fmol transferrin bound to GT vesicles prepared from basal cells, and 62 fmol bound to GT vesicles prepared from insulin-treated cells. This result was representative of four separate experiments, in which there was a  $48 \pm 5\%$  (SEM) decrease in transferrin receptor binding to the GT vesicles from insulin-treated cells. Transferrin binding to GT vesicles immunoadsorbed to antibody-coated *S. aureus* cells was not observed when Triton X-100 was omitted from the assay (data not shown). This observation is con-



**Figure 2.** Time course of insulin stimulation of PMP-BSA binding to cell surface receptors. Cells were incubated at 37°C with insulin (100 nM, final) for 0–20 min, followed by rapid cooling of the cells, and measurement of specific binding of PMP-BSA to cells at 4°C, as described in Materials and Methods. The results shown are the average of triplicate determinations and are representative of results obtained in three independent experiments.



**Figure 3.** IGF-II receptor and GT content of microsomal membranes prepared from basal and insulin-treated cells. Cells were incubated at 37°C with vehicle or insulin (100 nM, final) for 20 min, followed by rapid cooling of the cells, homogenization, and isolation of microsomal membranes, as described in Materials and Methods. Increasing amounts of microsomal membranes from basal cells (lanes 1–4) and insulin-treated cells (lanes 5–8) were subjected to SDS-PAGE, immunoblotting, and autoradiography. 0.875 (lanes 1 and 5), 1.75 (lanes 2 and 6), 3.5 (lanes 3 and 7), and 7% (lanes 4 and 8) of the microsomal membranes (~300 µg total protein) from 10-cm dishes of basal and insulin-treated cells were applied to the gel. The upper half of the immunoblot was incubated with a 1:10,000 dilution of rabbit antiserum against the bovine manose 6-phosphate receptor, and the lower half of the immunoblot was incubated with 0.5 µg/ml affinity-purified antibodies against the GT. Both halves were then incubated with <sup>125</sup>I-goat anti-rabbit IgG. Film exposure was for 17 h. Similar results were obtained with two other 10-cm plates prepared on the same day.

sistent with the binding site of the transferrin receptor being oriented towards the lumen of the vesicle.

#### **Effect of Insulin on the Subcellular Distribution of IGF-II Receptors**

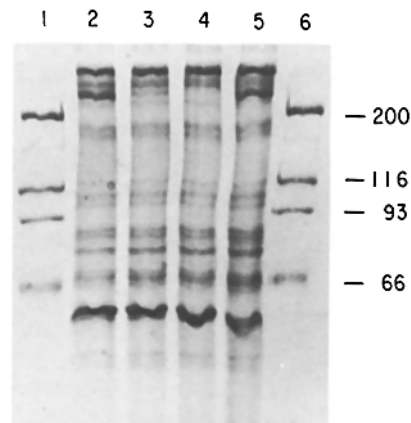
Insulin elicits translocation of IGF-II receptors in rat adipocytes (Oka et al., 1984; Wardzala et al., 1984). We used <sup>125</sup>I-PMP-BSA, a ligand developed for study of the manose 6-phosphate receptor (Braulke et al., 1987), to examine the effect of insulin on surface IGF-II receptors in 3T3-L1 adipocytes. The IGF-II receptor and cation-independent manose 6-phosphate receptor are the same protein (see Discussion). The time course of insulin stimulation of <sup>125</sup>I-PMP-BSA binding to surface receptors is shown in Fig. 2. When 3T3-L1 adipocytes were exposed to insulin, there was a maximal 2.5-fold increase in the capacity of cells to bind <sup>125</sup>I-PMP-BSA. In three independent experiments, the half-time for insulin action at 37°C was 2.3 ± 0.4 min (SEM), and insulin elicited a 2.5–4.5-fold stimulation in the amount of <sup>125</sup>I-PMP-BSA bound to cell surface receptors. Scatchard analysis of PMP-BSA binding demonstrated that the insulin-induced increase was due to an increase in the number of cell surface receptors (11.5 [basal] vs. 40 fmol/well [insulin]), with little effect on the value of the dissociation constant (1.8 nM [basal] vs. 1.3 nM [insulin]; results not shown). Thus, translocation of IGF-II receptors most likely also occurs in 3T3-L1 adipocytes.

Further evidence for insulin-stimulated translocation of IGF-II receptors was obtained by comparing the IGF-II re-

ceptor content of the microsomal membranes prepared from basal and insulin-treated cells. Shown in Fig. 3 is an autoradiograph of an immunoblot of increasing amounts of the microsomal membranes prepared from basal (Fig. 3, lanes 1–4) and insulin-treated cells (Fig. 3, lanes 5–8). In this experiment, over the range where the signal in immunoblotting was proportional to the amount of sample, there was 35% less IGF-II receptor in the microsomal membranes prepared from insulin-treated cells as compared with the corresponding fraction prepared from basal cells (Fig. 3, compare lane 2 vs. 6 and lane 3 vs. 7). These results indicate that insulin elicited a decrease in the amount of intracellular IGF-II receptors, which correlates with the insulin-stimulated increase in <sup>125</sup>I-PMP-BSA binding to cell surface receptors (Fig. 2). From a number of experiments of this type, the average reduction in the amount of intracellular IGF-II receptors in the microsomal membrane fraction in response to insulin was 16 ± 3% (n = 18; SEM). For comparison, in the experiment shown in Fig. 3 the GT content was also examined. Insulin elicited a 70% decrease in the amount of GT in the microsomal membranes (Fig. 3, compare lane 1 vs. 5 and lane 2 vs. 6).

#### **Localization of IGF-II Receptors in the Intracellular GT Vesicles**

We investigated whether IGF-II receptors are located in the

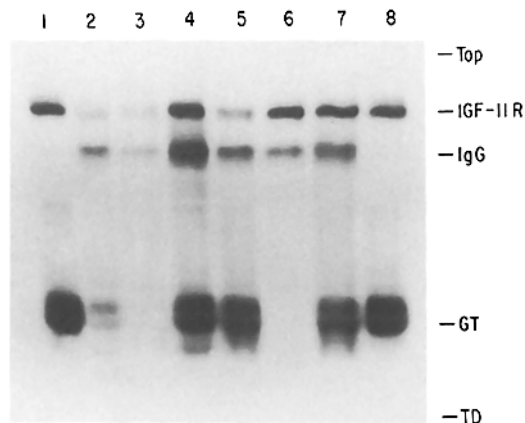


**Figure 4.** Immunoadsorption of the IGF-II receptor from solubilized GT vesicles. GT vesicles were isolated from the microsomal fraction of 16 10-cm plates of basal cells by immunoadsorption onto *S. aureus* cells, and then solubilized with 300 µl 1% C<sub>12</sub>E<sub>8</sub> in 150 mM NaCl, 20 mM Hepes, 2 mM MgSO<sub>4</sub>, pH 7.4. The C<sub>12</sub>E<sub>8</sub> extract (37 µl; 20 µg protein; equivalent of two 10-cm plates) was incubated for 2 h at room temperature with 10 µl *S. aureus* cells precoated with 100 µg nonimmune rabbit IgG (lane 2), 10 µl of rabbit antiserum against the C-peptide of the bovine manose 6-phosphate receptor (lane 3), or 10 µl of rabbit antiserum against the rat liver IGF-II receptor (lane 4). The supernatants from the immunoadsorptions were diluted in SDS sample buffer, samples (equivalent of 0.6 of a 10-cm plate) were subjected to SDS-PAGE as described in Materials and Methods, and the gel was stained with Coomassie blue. An equivalent amount of C<sub>12</sub>E<sub>8</sub> extract of GT vesicles which had been untreated was also run (lane 5). The standard proteins (150 ng each protein [Bio-Rad Laboratories]; molecular weights indicated at right ×10<sup>3</sup>) are shown in lanes 1 and 6. Immunoadsorption of the C<sub>12</sub>E<sub>8</sub> extract with *S. aureus* cells precoated with rabbit antiserum against the rat liver IGF-II receptor was performed in two other experiments, with identical results.

intracellular GT vesicles. The protein composition of GT vesicles was examined by solubilization of the vesicle proteins with octaethylene glycol dodecyl ether ( $C_{12}E_8$ ), resolution by SDS-PAGE, and staining with Coomassie blue (Fig. 4). The  $C_{12}E_8$  extract of the vesicles contains a major polypeptide of  $M_r$  230,000 (Fig. 4, lane 5). Incubation of the  $C_{12}E_8$  extract with *S. aureus* cells coated with either antibodies against the rat liver IGF-II receptor (Fig. 4, lane 4) or antibodies against the C-peptide of the bovine mannose 6-phosphate receptor (Fig. 4, lane 3) resulted in a selective depletion of this protein. In contrast, there was no depletion of any protein when the  $C_{12}E_8$  extract was incubated with *S. aureus* cells coated with nonspecific rabbit IgG (Fig. 4, lane 2). These results demonstrate that the IGF-II receptor, which has been previously shown to have an apparent  $M_r$  of 220,000 in a nonreducing gel (Rechler and Nissley, 1985), is a major component of the intracellular GT vesicles. This conclusion is supported by the finding that the IGF-II receptor was also detected on an immunoblot of the  $C_{12}E_8$  extract of GT vesicles with antibodies against the IGF-II receptor (data not shown). Parenthetically, we note that the Coomassie blue-stained band in Fig. 4 with an apparent molecular mass in the range of the GT ( $\sim 50,000$ ) is not the GT, since during  $C_{12}E_8$  solubilization of the vesicle proteins the GT remains bound to its antibodies on the *S. aureus* cells (Brown et al., 1988).

To characterize further the intracellular GT vesicles, immunoadsorption of the membranes in the microsomal fraction was performed with GT C-peptide antibodies and antibodies against the C-peptide of the IGF-II receptor (Fig. 5). Immunoadsorption of the microsomal fraction with *S. aureus* cells coated with GT C-peptide antibodies (Fig. 5, lane 3) resulted in a 70% depletion of IGF-II receptor, as well as an 88% depletion of GT, when compared with the original supernatant (Fig. 5, lanes 1 and 8). In five separate experiments, there was an  $82 \pm 4\%$  (SEM) depletion of IGF-II receptors and a  $90 \pm 3\%$  (SEM) depletion of GT. This result reinforces the conclusion drawn from the data in Fig. 4 that the intracellular GT vesicles also contain IGF-II receptors. Immunoadsorption of the microsomal fraction with *S. aureus* cells coated with antibodies to the C-peptide of the IGF-II receptor (Fig. 5, lane 2) resulted in a 70% depletion of both IGF-II receptor and GT. In three separate experiments, there was a  $77 \pm 5\%$  (SEM) depletion of IGF-II receptor and a  $76 \pm 10\%$  (SEM) depletion of GT. Thus, almost all of the vesicles containing GT also contain IGF-II receptor. When the microsomal membrane fraction was incubated with *S. aureus* cells coated with nonimmune IgG, there was no loss of either IGF-II receptor or GT immunoreactivity (Fig. 5, lane 4).

The amounts of *S. aureus* cells and antibody that were used in the experiment shown in Fig. 5 are saturating, since similar results were obtained when 1-ml aliquots of the microsomal fraction were immunoadsorbed with 4  $\mu$ l cells coated with 15  $\mu$ g of the appropriate antibodies (results not shown). The immunoreactive band in Fig. 5, lanes 2–7, of  $M_r$  150,000 is due to IgG that dissociated from the *S. aureus* cells into the supernatant during the 2-h incubation. This band is absent from Fig. 5, lanes 1 and 8, which contain microsomal fractions that were not immunoadsorbed. In addition, when immunoblots were probed with  $^{125}I$ -labeled goat antibodies against rabbit IgG in the absence of prior in-



**Figure 5.** Immunoadsorption of GT-containing vesicles and IGF-II receptor-containing vesicles from the microsomal fraction. 1-ml aliquots of the microsomal fraction (12.5% of microsomal fraction from a basal 10-cm plate) were incubated at room temperature for 2 h with 2  $\mu$ l *S. aureus* cells coated with 6  $\mu$ g affinity-purified antibodies against the C-peptide of the rat IGF-II receptor (lanes 2 and 5), 6  $\mu$ g affinity-purified GT C-peptide antibodies (lanes 3 and 6), or 6  $\mu$ g nonimmune rabbit IgG (lanes 4 and 7). Lanes 1 and 8 contain samples of equivalent amounts of untreated microsomal fraction. In addition, 0.1% Triton X-100 was present during the immunoadsorption of the samples in lanes 5–7. The vesicles adsorbed to the antibody-coated *S. aureus* cells were then pelleted, and the resulting supernatants were subjected to SDS-PAGE and immunoblotting, as described in Materials and Methods and in the legend to Fig. 3. An 18-h exposure of the autoradiogram is shown. Similar results on the parallel immunoadsorption of IGF-II receptor and GT from the microsomal fraction by incubation with *S. aureus* cells coated with affinity-purified GT C-peptide antibodies were obtained in four additional experiments. Similar results on the parallel immunoadsorption of IGF-II receptor and GT from the microsomal fraction by incubation with *S. aureus* cells coated with affinity-purified antibodies against the C-peptide of the IGF-II receptor were obtained in two additional experiments.

incubation with antibodies against the GT or IGF-II receptor, the band of  $M_r$  150,000 was the only one present (data not shown).

To be certain that the results shown in Fig. 5, lanes 2 and 3, were not due to cross-reactivity of the GT antibodies and IGF-II receptor antibodies with the IGF-II receptor and GT, respectively, parallel immunoadsorptions were performed in the presence of 0.1% Triton X-100. Under this condition, where the membranes in the microsomal fraction were solubilized, immunoadsorption of the microsomal fraction with *S. aureus* cells coated with antibodies against the C-peptide of the IGF-II receptor resulted in a 45% loss of IGF-II receptor, but no loss of GT (Fig. 5, lane 5). Conversely, there was a 94% depletion of GT, but no loss of IGF-II receptor, when the microsomal fraction was incubated with *S. aureus* cells coated with GT C-peptide antibodies (Fig. 5, lane 6). Again, immunoadsorption of the microsomal fraction with *S. aureus* cells coated with nonimmune IgG resulted in no loss of either GT or IGF-II receptor (Fig. 5, lane 7). These results indicate that the antibodies against the C-peptides of the GT and IGF-II receptor exhibit no cross-reactivity for the other protein. In another control experiment, *S. aureus* cells coated with antibodies against the C-peptide of the IGF-II receptor did not adsorb purified human erythrocyte GT reconstituted into membranes, under conditions where *S. aureus* cells

coated with GT C-peptide antibodies resulted in quantitative adsorption (results not shown).

## Discussion

We have shown that insulin elicits the translocation of transferrin receptors and IGF-II receptors in 3T3-L1 adipocytes, and that intracellular GT vesicles contain these two additional insulin-responsive membrane proteins. In our earlier study, in which the steady-state distribution of  $^{125}\text{I}$ -transferrin between the surface and interior of intact cells was measured, we concluded that insulin increased the number of surface transferrin receptors and reduced the number of intracellular transferrin receptors, due to an increase in the rate constant for externalization of the receptor (Tanner and Lienhard, 1987). In this study, subcellular fractionation and measurement of transferrin binding to soluble receptors has provided further evidence for translocation of transferrin receptors in response to insulin (Table I). Moreover, all of the transferrin receptors in the microsomal fraction prepared from both basal and insulin-treated cells were immunoprecipitated by *S. aureus* cells coated with GT C-peptide antibodies (Table I).

Previously, we found that the amount of surface transferrin receptors on a 35-mm dish of 3T3-L1 adipocytes increases by  $\sim 12$  pmol in response to insulin (Tanner and Lienhard, 1987). Since the number of cells on a 10-cm plate is about six times greater, the expected increase for a 10-cm plate is  $\sim 72$  pmol. By comparison, insulin treatment of intact cells resulted in a reduction in the amount of transferrin receptors in the microsomal membranes from a 10-cm plate by 120 pmol (Fig. 1). This calculation indicates that all of the insulin-responsive intracellular transferrin receptors are contained in the microsomal fraction, as is also the case for the insulin-responsive GT (Brown et al., 1988).

An estimate of the number of transferrin receptors in the GT vesicles can be made in the following way. According to electron microscopy, the GT vesicles are  $\sim 50$  nm in diameter (Biber and Lienhard, 1986). The molecular weight of the phospholipid in a vesicle of this size is  $\sim 1.5 \times 10^7$  (Huang and Mason, 1978). Since 11  $\mu\text{g}$  of membrane lipid are present in the GT vesicles isolated from the microsomal fraction of a 10-cm plate of basal cells (Brown et al., 1988), there are  $\sim 0.7$  pmol of vesicles in the microsomal fraction. Since this fraction contains 0.27 pmol of transferrin receptors (Fig. 1), on the average only one vesicle in three contains a transferrin receptor. By comparison, the average vesicle contains eight GT (Brown et al., 1988).

In contrast to rat adipocytes which possess no IGF-I receptors (Massagué and Czech, 1982), 3T3-L1 adipocytes have high levels of both IGF-I and IGF-II receptors, as demonstrated by affinity labeling (Massagué and Czech, 1982). IGF-II binds with high affinity to the IGF-II receptor, and with lower affinity to the IGF-I receptor (Rechler and Nissley, 1985). For this reason, binding of  $^{125}\text{I}$ -PMP-BSA, rather than  $^{125}\text{I}$ -IGF-II, was used to monitor cell surface IGF-II receptors. Recent studies have proven that the IGF-II receptor is identical to the cation-independent mannose 6-phosphate receptor (Roth, 1988). Insulin elicited a redistribution of IGF-II receptors in 3T3-L1 adipocytes. There was a 2.5–4.5-fold increase in the number of surface receptors (Fig. 2), and an average 16% decrease in the amount of intracellular

IGF-II receptors in microsomal membranes (Fig. 3). These results are similar to those obtained in rat adipocytes, where insulin treatment resulted in a 2.5–10-fold increase in the amount of surface IGF-II receptors, and a 17–40% decrease in intracellular IGF-II receptors (Oppenheimer et al., 1983; Oka et al., 1984; Wardzala et al., 1984; Appell et al., 1988).

Given that the microsomal membranes contain an insulin-responsive pool of intracellular IGF-II receptors (Fig. 3), and that an average of 80% of the IGF-II receptors in the microsomal fraction were immunoadsorbed by incubation with *S. aureus* cells coated with GT C-peptide antibodies (Fig. 5), the IGF-II receptors localized in the GT vesicles most likely constitute this insulin-responsive pool. It is unlikely that immunoadsorption of the IGF-II receptor from the microsomal fraction by incubation with *S. aureus* cells coated with GT C-peptide antibodies could have resulted from nonspecific sticking of separate IGF-II receptor-containing vesicles. The GT vesicles isolated from the microsomal fraction are highly enriched; only 15  $\mu\text{g}$  vesicle protein is obtained from 300  $\mu\text{g}$  of membrane protein present in the microsomal fraction from a 10-cm plate of basal cells (Brown et al., 1988). We cannot, of course, rigorously exclude the possibility that specific fusion of separate vesicles containing IGF-II receptor with ones containing GT occurred during the isolation procedure.

A rough estimate of the average IGF-II receptor content per vesicle can be made in the following way. On the basis of visual estimation of the intensity of the Coomassie blue stain in the IGF-II receptor band relative to the intensity of 150 ng of each standard protein (Fig. 4), there is  $\sim 250$  ng of IGF-II receptor in the GT vesicles from the microsomal fraction of a 10-cm plate of basal cells. The molecular mass of the receptor calculated from its deduced amino acid sequence is 275 kD (Lobel et al., 1988; Oshima et al., 1988). Consequently there are 0.9 pmol of receptor in the GT vesicles, which corresponds to an average of 1.3 receptors per GT vesicle (see above). A content of at least one IGF-II receptor per GT vesicle is consistent with the results shown in Fig. 5, where *S. aureus* cells coated with antibodies against

Table II. Subcellular Distributions of the GT, Transferrin Receptor, and IGF-II Receptor

	Cell surface		Crude plasma membranes		Microsomal membranes	
	Basal	Insulin	Basal	Insulin	Basal	Insulin
	%	%	%	%	%	%
GT	15	43	50	75	50	25
Transferrin receptor	15	30	66	80	34	20
IGF-II receptor	1.7	5.9	73	77	27	23

The values, given as a percentage of the total, are from the following sources. (GT) Cell surface, by quantitative immunoelectron microscopy (Blok et al., 1988); crude plasma membranes and microsomal membranes, by immunoblotting (Brown et al., 1988). (Transferrin receptor) Cell surface, by steady-state distribution of  $^{125}\text{I}$ -transferrin (Tanner and Lienhard, 1987); crude plasma membranes and microsomal membranes, from Table I. (IGF-II receptor) Cell surface, calculated on the assumption that the 27% of receptor in basal microsomal membranes equals 1.1 pmol (see Discussion) and the pmol of surface receptor as measured by binding of  $^{125}\text{I}$ -PMP-BSA (see Results); crude plasma membranes and microsomal membranes, by immunoblotting (see Results and Fig. 3), with the value for insulin crude plasma membranes obtained by difference.

the C-peptide of the rat IGF-II receptor adsorbed 70% of both the IGF-II receptor and the GT present in the microsomal fraction.

This rough estimate of intracellular IGF-II receptors also indicates that the decrease in IGF-II receptors in the microsomal membranes in response to insulin can account for the increase in surface receptors as determined by PMP-BSA binding. The decrease in the microsomal membranes was  $\sim 0.18$  pmol/10-cm plate (calculated from 0.9 pmol in the GT vesicles, 82% immunoprecipitation of the IGF-II receptor in the microsomal fraction with the GT vesicles, and a 16% decrease in the IGF-II receptor content of the microsomal membranes by immunoblotting), whereas the increase in surface receptors was 0.17 pmol/10-cm plate (calculated from an increase of 28.5 fmol/35-mm dish measured by Scatchard analysis [see Results]).

Table II summarizes the effects of insulin on the subcellular distributions of the GT, transferrin receptor, and IGF-II receptor in 3T3-L1 adipocytes. As discussed above, insulin stimulated a two- to threefold increase in the level of each protein at the cell surface, and in each case the decrease in the content of the protein in the microsomal membranes can account for the increase at the cell surface. Although 85–100% of these three membrane proteins in the microsomal fraction are contained in the GT vesicles (see Table I and Fig. 5), insulin's effect on the content of the proteins in this fraction was not uniform. The effect of insulin varied from a 50% decrease in the GT content to only a 16% decrease in the IGF-II receptor content. A possible explanation for this fact is that the GT vesicles consist of fragmented *trans*-Golgi reticulum (see below) and that translocation of these proteins to the plasma membrane involves a sorting step at the stage in which vesicles destined to fuse with the plasma membrane bud from this reticulum. In response to insulin, the GT may have a higher probability of being incorporated into these shuttle vesicles than does the IGF-II receptor.

A vesicle population enriched in GT has also been prepared from rat adipocytes (James et al., 1987). The procedure involved isolation of a low-density microsomal fraction, followed by sucrose gradient centrifugation, and agarose gel electrophoresis. IGF-II receptors were present in the agarose fractions enriched in GT. However, it was unclear as to whether IGF-II receptors and GT were located in the same vesicles, or whether these two proteins were contained in different vesicle populations of the same size that comigrated on agarose gel electrophoresis.

Results in two recently published papers suggest that insulin regulates the subcellular distribution of IGF-II receptors and GT by different mechanisms. In chloroquine-treated rat adipocytes, insulin stimulation of hexose transport and GT translocation is unaffected, but the insulin-induced increase in surface IGF-II receptors is markedly attenuated (Oka et al., 1987). Chloroquine treatment, however, does not affect the basal levels of surface IGF-II receptors. Although these results show that the IGF-II receptor and the GT behave differently in the presence of chloroquine, they do not indicate whether the trafficking of these proteins differs in its absence. The dissociation of lysosomal enzymes from intracellular IGF-II receptor is inhibited in the presence of chloroquine (reviewed by von Figura and Hasilik, 1986), and this may affect the sorting of the insulin-responsive pool of intracellular IGF-II receptors. In a second study (Appell et al., 1988), it was found that in rat adipocytes the time courses

for both the insulin-induced appearance of surface IGF-II receptors and the reversal of this effect upon insulin withdrawal are each more rapid than the corresponding time courses for the insulin stimulation of the rate of glucose transport and its reversal. However, recent results indicate that these different time courses do not necessarily mean that the insulin regulation of the translocation of the GT and IGF-II receptor differ. In 3T3-L1 adipocytes the appearance of GT at the cell surface in response to insulin occurs about twice as rapidly as does the increase in the rate of glucose transport (Gibbs et al., 1988). It remains to be determined whether the time course of the insulin-stimulated translocation of GT to the cell surface parallels that of the transferrin receptor and IGF-II receptor.

Recently, a monoclonal antibody was raised against partially purified GT vesicles from rat adipocytes (James et al., 1988). This antibody appears to react with the GT in rat fat and muscle, but not with the GT in rat brain or human erythrocytes. On this basis it has been proposed that there is a unique GT in insulin-responsive tissues. The antibodies that we have used for immunoadsorption of the GT vesicles were raised against the C-peptide of the human erythrocyte/rat brain GT (Davies et al., 1987). Sequencing of a cDNA has shown that mouse 3T3-L1 adipocytes possess a GT that is very similar to those in erythrocyte and brain, with the same carboxy-terminal sequence (Reed, B. C., D. Shade, F. Alperovich, and M. Vang, manuscript in preparation). It remains to be determined whether 3T3-L1 adipocytes possess a second type of GT, and if so, whether our GT C-peptide antibodies react with it and whether the insulin-responsive pool of the two types of GT reside in the same vesicle population.

The cellular locations of GT in ultrathin cryosections of basal and insulin-treated 3T3-L1 adipocytes have been examined (Blok et al., 1988). In response to insulin, the GT content of an intracellular compartment drops from 55 to 29% of the total GT, with the GT content of the plasma membrane correspondingly increased. Based on morphological criteria, the insulin-responsive intracellular GT appear to be in the *trans*-Golgi reticulum, an organelle in which secretory proteins and proteins destined for the plasma membrane and lysosomes are sorted (Griffiths and Simons, 1986). However, this organelle cannot be unequivocally distinguished by its morphology (Blok et al., 1988; van Deurs et al., 1988) from the endosomal compartment in which ligands uncouple from their receptors (CURL) (Geuze et al., 1983). In this regard, the intracellular GT vesicles that contain the transferrin receptor are in communication with the extracellular medium. When 3T3-L1 adipocytes were incubated for 1 h at 37°C with  $^{125}\text{I}$ -transferrin, the GT vesicles subsequently immunoadsorbed from the microsomal fraction contained receptor-bound  $^{125}\text{I}$ -transferrin (data not shown). Future research will be directed at identification of the organelle(s) from which the GT vesicles are derived.

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us with the amino acid sequence of the cytoplasmic domain of the rat IGF-II receptor before its publication, and we are indebted to T. Ciardelli for synthesis of the carboxy-terminal peptide of the rat IGF-II receptor.

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