Insulin Regulation of the Two Glucose Transporters in 3T3-L1 Adipocytes

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The amounts of the brain type and muscle type glucose transporters (designated Glut 1 and 4, respectively) in 3T3-L1 adipocytes have been determined by quantitative immunoblotting with antibodies against their carboxyl-terminal peptides. There are about 950,000 and 280,000 copies of Glut 1 and 4, respectively, per cell. Insulin caused the translocation of both types of transporters from an intracellular location to the plasma membrane. The insulin-elicted increase in cell surface transporters was assessed by labeling the surface transporters with a newly developed, membrane-impermeant, photoaffinity labeling reagent for glucose transporters. The increases in Glut 1 and 4 averaged 6.5- and 17-fold, respectively, whereas there was a 21-fold in hexose transport. These results indicate that the translocation of Glut 4 could largely account for the insulin effect on transport rate, but only if the intrinsic activity of Glut 4 is much higher than that of Glut 1. The two transporters are colocalized intracellularly: vesicles (average diameter 72 nm) isolated from the intracellular membranes by immunosorbation with antibodies against Glut 1 contained 95% of the Glut 4 and, conversely, vesicles isolated with antibodies against Glut 4 contained 85% of the Glut 1.

We have previously shown that a pool of the Glut 1 in 3T3-L1 adipocytes is located in intracellular membranes and that insulin causes the translocation of a portion of this intracellular Glut 1 to the plasma membrane. However, the increase in Glut 1 at the cell surface in response to insulin, which we have assessed by quantitative immunoelectron microscopy (8), by labeling of surface Glut 1 with galactose oxidase and [3H]borohydride (2), and by the relative Glut 1 content of a plasma membrane fraction (9), is only 2-3-fold, much less than the increase in the hexose transport rate. We have also described the isolation of membranous vesicles that contain the insulin-responsive intracellular Glut 1 and the characterization of these vesicles (10).

The present study was undertaken to define the role of Glut 4 in insulin-stimulated hexose transport in 3T3-L1 adipocytes. Through the use of a newly developed immunological method, the absolute amounts of the two transporters in 3T3-L1 adipocytes has been determined. By means of a novel membrane-impermeant, photoactivated affinity-labeling reagent for glucose transporters (26), and also by subcellular fractionation, the insulin-elicted increases in both Glut 1 and Glut 4 at the cell surface have been assessed. Finally, the vesicles containing the intracellular Glut 1 have been shown to contain also the intracellular Glut 4.

EXPERIMENTAL PROCEDURES

Materials—Trition-labeled 2-N-(1-aza-2,2,2-trifluoroethyl)benzoyl)-1,3-bis[(D-mannos-4-yloxy)-2-propylamine (ATB-BMCPA) (about 10 Ci/mmol) and the unlabeled compound were prepared by procedures reported elsewhere (26).

Cell Culture—3T3-L1 fibroblasts were cultured and differentiated into adipocytes on 3.5- and 10-cm plates as described (1). The cells were incubated in serum-free Dulbecco's modified Eagle's medium for 2 h prior to use.

Antibodies—Rabbit antiserum against the carboxyl-terminal peptide of Glut 1 (residues 477-492) was a gift from Dr. Stephen A. Baldwin, Royal Free Hospital School of Medicine, London (11). Antibodies against Glut 1 were affinity purified from this serum, as described in Ref. 12. Antibodies were then affinity purified by chromatography of the serum on immobilized peptide (17). Before these antibodies against Glut 4 were available, some initial experiments were performed with the monoclonal antibody 1F8 against Glut 4 (4), kindly provided by Dr. David E. James of Washington University School of Medicine.

Immunoblotting—SDS samples were prepared in the sample buffer with protease inhibitors, as given in Ref. 13. For determination of the transporter contents of 35-mm plates, cells were scraped into 400 µl of sample buffer, and the DNA in the lysate was sheared by passages of the lysate through a syringe needle. SDS-polyacrylamide gel electrophoresis was carried out on small 10% slab gels, and the polypeptides were electrophotoehoreically transferred to nitrocellulose, as de-

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terminal peptide in the blocking medium. The nitrocellulose was then washed five times with buffer A. For Glut 4 immunoblots, the nitrocellulose was blocked with 5% nonfat dry milk (Carnation) in PBS and then treated with 5 μg/ml antibodies against the carboxyl-terminal peptide of Glut 4 in 1% milk/PBS. After five washes with buffer A, the blot was exposed to 0.07 μCi/ml [3H]-labeled second antibody in 1% dry milk/PBS for 3 h and then washed five times with buffer A. The nitrocellulose blots were subjected to autoradiography, and radioactivity in the Glut bands was measured by cutting and counting, as detailed in Ref. 13. In the case of the experiment in Fig. 6, the procedure used for Glut 4 was also used for Glut 1 because these samples gave a higher background with some nonspecific bands when blocked with albumin. In this case the antibodies against the carboxyl-terminal peptide of Glut 1 were present at 5 μg/ml on the nitrocellulose. Under "Results," human erythrocyte membranes, prepared by the method in Ref. 14, and rat low density microsomes, prepared as in Ref. 15, served as standards for Glut 1 and 4, respectively. The transporter content of the microsome preparation, as determined by D-glucose-inhibitable cytochalasin B binding (3), was 40 pmol/mg protein. This value was confirmed by comparison of the [3H] content of this preparation through immunoblotting with that of a different one kindly provided by Dr. Ian Simpson at the National Institutes of Health, for which he had determined a transporter content of 61 pmol/mg by cytochalasin B binding.

**ATB-BMPA Labeling**—Plates of cells (25 mm) were washed with Krebs-Ringer-phosphate buffer (KRP buffer) (136 mM NaCl, 4.7 mM KCl, 1.25 mM CaCl2, 1.25 mM MgSO4, 5 mM sodium phosphate, pH 7.4) and incubated in 1 ml of KRP at 37 °C in the presence or absence of insulin (1 μM) for 15 min. Under these conditions the full stimulation of hexose transport occurs (16). Following this stimulation, the cells were washed quickly with KRP at 25 °C and then incubated with 250 μl of KRP containing 400 μCi of [3H]ATB-BMPA (about 180 μM) for 2 min at 25 °C. The dishes were then irradiated for 3 min on a wire shelf placed 5 cm from the bottom in a Rayonet KRP-100 reactor with RPR-3000 A lamps turned on its side. Control experiments showed that there was no reversal of the insulin effect on transport during this 5 min of incubation under these conditions and that the half-time for photolysis of the diazirine functional group under these conditions was 1.0 min, as measured spectrophotometrically by the decomposition of the precursor 4-(1-azirino-2,2,2-trifluoroethyl)benzoate (Bachem). The irradiated cells were washed three times with 250 μl of KRP and solubilized in 1 ml of 1% Triton X-100/0.1% deoxycholate, 20 mM Tris, 50 mM sodium fluoride, 1 pM pepstatin A, and 10 μM L-trans-epoxysuccinyl-leucylamido-(3-methyl)butane (10 mM) (Tocris). The cell extracts were centrifuged at 190,000 × g, for 60 min, and 1.4 ml of the supernatant was used for immunoprecipitation of the Glut 4 at 4 °C. Antibodies against the carboxyl terminus (7.5 μg) were incubated with the supernatant for 1.5 h, and the immune complexes were collected by rotating the mixture with 10 μl of protein A-Sepharose (Pharmacia LKB Biotechnology Inc.) for 2 h. Glut 1 was then isolated from 1.2 ml of the Glut 4-depleted supernatant by immunoprecipitation with antibodies against the carboxyl terminus of Glut 1 in 1% C6E/PBS, as described by this same group (17). In both cases the protein-A-Sepharose immunoadsorbate was washed three times with 1% C6E/PBS and one time with 0.1% C6E/PBS. The transporter was released by the addition of 30 μl of SDS sample buffer containing 8 M urea and 600 ng each of protein low molecular weight standards (Bio-Rad). These samples were then run on 10% acrylamide gels; the gels were stained with Coomassie Blue, and the individual lanes were cut into 4-mm slices and placed in 10 ml of 6% Protosol in Econofluor (both from Du Pont-New England Nuclear). The gel slices were solubilized by shaking overnight at 37 °C and their "H content was measured. The "H values for the transporter-containing slices were corrected for the background by means of the values for the adjacent slices as described (2).

Several control experiments in which the relative amounts of Glut 1 and of Glut 4 were quantitated by immunoblotting were carried out to validate this protocol. The extents of immunoprecipitation of Glut 1 and 4 from the C6E supernatant were determined by comparing the amount of each transporter remaining in the supernatant after immunoadsorption with the amount in the unadsorbed supernatant. About 70% of the Glut 1 and 85% of the Glut 4 were immunoprecipitated. Only a fraction of the adsorbed transporters (in each case about 30% of the total in the unadsorbed supernatant) was released from the protein A-Sepharose into the SDS-urea sample buffer. In two different immunoadsorption experiments both Glut 1 and of Glut 4 in these SDS-urea samples derived from basal and insulin-treated cells were compared and found to be the same.

In some experiments, which are not reported in Table I, the amount of label found in Glut 1 and 4 was about one-third the values given in the table. The basis for the low label was traced to the loss of the transporters in the SDS-urea samples by adsorption onto the walls of colored plastic microfuge tubes to which they had been transferred. To avoid this problem, we now leave the SDS-urea sample over the protein A-Sepharose in the clear 1.5-ml microfuge tubes (USA Scientific Plastics or Sarstedt) used for immunoprecipitation.

**Hexose Transport** (1, 16)—For determinations at the same time as the ATB BMPA labeling experiment 35 mm plates were washed with and placed in 1 ml of KRP at 37 °C with or without 1 μM insulin for 15 min. Transport was initiated by addition of 50 μl of [2-14C]deoxyglucose, such that the final concentrations were 0.5 μCi/ml and 50 μM 2-deoxyglucose. Uptake was terminated after 5 min by rapid chilling in ice-cold PBS, and the cells were washed four times with buffer A. The labeled cells were assayed by comparison of the [3H] content of this preparation through immunoblotting with that of a different one kindly provided by Dr. Ian Simpson at the National Institutes of Health, for which he had determined a transporter content of 61 pmol/mg by cytochalasin B binding.

**ATB-BMPA**—The ATB-BMPA was added 5 min before the initiation of uptake. Since the nonspecific association of 2-deoxyglucose with the monolayers was found to increase slightly at higher concentrations of ATB-BMPA, the uptake was assayed in the presence of cytochalasin B at several different concentrations of ATB-BMPA across the range of interest, and these values were subtracted from the corresponding values of the rate in the presence of ATB-BMPA alone. Uptake by insulin-stimulated cells was again for 5 min, but because the inhibited rates for basal cells were low, uptake by these was for 10 min.

**Subcellular Fractions**—The following method was employed to assess translocation of the transporters. Plates (10 cm) of 3T3-L1 adipocytes in serum-free Eagle's medium at 37 °C were treated with 160 μM insulin for 15 min or left in the basal state. Subsequent operations were done with basal and insulin-treated cells, in parallel at 4 °C. The cells were washed twice with 250 mM sucrose, 20 mM Hepes, 1 mM EDTA, pH 7.4, and then scraped into 1.5 ml of 250 mM sucrose, 20 mM Hepes, 1 mM EDTA, pH 7.4, with protease inhibitors dithiothreitol, 10 mM sodium fluoride, 1 μM pepstatin A, and 10 μM L-trans-epoxysuccinyl-leucylamido-(3-methyl)butane (1 mM). The cells from two plates were homogenized together in a 55-ml Wheaton tissue grinder (No. 358054) at setting 6 for 7 up-and-down strokes. Portions of the homogenates (2.2 ml) of basal and insulin-treated cells were loaded on 10-ml linear gradients of 15 to 37% (w/w) sucrose in 20 mM Hepes, 1 mM EDTA, pH 7.4, and these were centrifuged at 35,000 rpm for 45 min (centrifuge setting) in the SW 41 rotor of a Beckman ultracentrifuge. Fractions (920 μl) were collected, and SDS samples of the fractions, as well as of the pellet and homogenate, were immunoblotted for Glut 1 and 4.

The procedures used for the preparation of the fraction containing intracellular membranes and the immunoadsorption of vesicles containing glucose transporters were those described in detail elsewhere (10). A brief outline is as follows. Cells were homogenized in 150 mM KCl, 20 mM Hepes, 2 mM MgSO4, pH 7.4, with protease inhibitors, and the homogenate was centrifuged at 16,000 × g, for 20 min. The 16,000 × g supernatant from basal cells contains about 50% of the total Glut 1 and 4 but only 6% of the plasma membrane; as a result the transporters from insulin-treated cells contain about 23% of the total (data not shown). Vesicles containing Glut 1 and 4 were adsorbed from this supernatant by incubation with formaldehyde-fixed Staph A cells coated with antibodies against the carboxyl terminus of Glut 1 or 4. After removal of the Staph A cells, unadsorbed membranes in the supernatant were recovered by sedimentation at 180,000 × g for 1 h.
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FIG. 1. **Specificity of the antibodies against Glut 1 and 4.** 3T3-L1 adipocytes were solubilized in 2% CHAPS with protease inhibitors and the Glut 1 and 4 were immunoprecipitated from separate portions of the CHAPS supernatant with the affinity purified antibodies against their carboxyl-terminal peptides, as described under "Experimental Procedures." As a control, the antibodies alone were adsorbed from the solubilization buffer onto protein A-Sepharose. The immunoadsorbates were solubilized in SDS sample buffer and blotted for Glut 1 (part A) and Glut 4 (part B). Lanes 1-4: 1, immunoprecipitate with antibodies to Glut 1, from 30% of a 35-mm plate; 2, immunoprecipitate with antibodies to Glut 4, from 30% of a 35-mm plate; 3, antibodies to Glut 1 alone; 4, antibodies to Glut 4 alone. The band present in all the lanes, which is at about M₅ 80,000, is the heavy-light chain of the antibodies (IgG). This figure and Figs. 2, 5-7 show the immunoblots for the entire 10% gels.

FIG. 2. **Quantitation of Glut 1 and 4 in 3T3-L1 adipocytes.** 3T3-L1 adipocytes (35-mm plates) on day 9 after differentiation were treated with 1 μM insulin or not for 15 min and then solubilized in SDS sample buffer, as described under "Experimental Procedures." Samples were then immunoblotted with antibodies against the carboxyl-terminal peptide of each. Since the carboxyl-terminal 20 amino acids of Glut 1 and 4 are the same at only four positions, which except for a dipeptide, are separated in the sequence (5), the antibodies against this region of the two transporters should not cross-react. In agreement with this expectation, when Glut 1 and 4 were immunoprecipitated from 3T3-L1 adipocytes and then each immunoprecipitate was immunoblotted for both transporters, only Glut 1 was detected in the immunoprecipitate with antibodies against Glut 1 and only Glut 4 was detected in the immunoprecipitate with antibodies against Glut 4 (Fig. 1). Thus, the antibodies against one transporter neither immunoprecipitated nor immunoblotted the other.

**RESULTS**

**Amounts of Glut 1 and 4 in 3T3-L1 Adipocytes**—The amounts of Glut 1 and 4 were determined by quantitative immunoblotting with antibodies specific for the carboxyl-terminal peptide of each. Since the carboxyl-terminal 20 amino acids of Glut 1 and 4 are the same at only four positions, which except for a dipeptide, are separated in the sequence (5), the antibodies against this region of the two transporters should not cross-react. In agreement with this expectation, when Glut 1 and 4 were immunoprecipitated from 3T3-L1 adipocytes and then each immunoprecipitate was immunoblotted for both transporters, only Glut 1 was detected in the immunoprecipitate with antibodies against Glut 1 and only Glut 4 was detected in the immunoprecipitate with antibodies against Glut 4 (Fig. 1). Thus, the antibodies against one transporter neither immunoprecipitated nor immunoblotted the other.

For the assay of Glut 1, the standards were human erythrocyte membranes, in which Glut 1 is known to constitute 5.2% of the protein by weight (14). The antibodies against the carboxyl-terminal peptide of Glut 1 should react equally well with the human and mouse Glut 1, since the sequence of this peptide is the same in the Glut 1s of these two species (5, 18). For Glut 4, the standards were rat adipocyte low density microsomes. The total transporter content of these was determined by cytochalasin B binding. Although these microsomes contain both Glut 1 and Glut 4, there is evidence that at least 90% of the transporter in these is Glut 4 (19, 20). We
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have confirmed the low percentage of Glut 1 by determining the Glut 1 content of the microsomes through immunoblotting, with human erythrocyte membranes as standards (Glut 1 85% of the total, data not shown). In this case also, the antibodies against the carboxyl-terminal peptide should react equally as strongly with the standard and the 3T3-L1 transporter, since the sequence of the carboxyl-terminal peptide of Glut 4 is the same in rat and mouse (4, 5).

Fig. 2 shows the results of an experiment in which the amounts of the two transporters were determined on day 9 after the initiation of differentiation. The values for Glut 1 and 4 were 215 and 45 ng, respectively, per 35-mm plate of 3T3-L1 adipocytes (2.1 × 10^6 cells; about 1.5 mg of total protein). The relative amounts of Glut 1 and 4 on days 8 through 12 after the initiation of differentiation were also determined by immunoblotting. Glut 1 was constant over this period, whereas Glut 4 increased moderately; its relative value was 1.0 (day 8), 1.4 (day 9), and 1.8 (days 10–12) (data not shown). Thus, the expected ratio of Glut 1 to Glut 4 in cells on days 10 to 12 is 215/(45 × 1.8)/1.4 or 3.8. In another experiment of the type presented in Fig. 1, performed 6 months later with cells on day 11, the amounts of Glut 1 and 4 were found to be 150 and 50 ng/35-mm plate, respectively, and consequently the ratio was 3.0. Thus, although both transporters are present in 3T3-L1 adipocytes, Glut 1 is the predominant one. Because of the increase in Glut 4 between day 8 and 10, the studies described below were carried out with adipocytes at days 10–12 after differentiation.

The data in Fig. 1 also show that the amounts of Glut 1 and 4 detected in cells where transport was fully stimulated by insulin were the same as in basal cells, and that insulin treatment does not alter the electrophoretic mobility of either transporter. In one experiment, the electrophoretic mobilities of Glut 1 and 4 on SDS-gel electrophoresis were directly compared by immunoblotting adjacent lanes from the same blot. The mobilities of the two transporters were the same and corresponded to a molecular weight of 55,000.

Translocation of the Glucose Transporters: Labeling of Surface Transporters—Holman and associates (26) have recently developed an improved membrane-impermeant, photactivated affinity labeling reagent for glucose transporters. This compound, ATB-BMPA, is a bis(D-mannose) derivative in which the two mannoses are linked by a propyl bridge between the oxygens at carbons 4 and in which the diazirine, 4-(1-azido-2,2,2-trifluorethyl)benzamido, is linked to the middle carbon of the propyl bridge. Photolysis of this compound generates a highly reactive carbene through the loss of N2 from the diazirine functional group.

3T3-L1 adipocytes were labeled with [3H]ATB-BMPA, and the glucose transporters were isolated by immunoprecipitation and gel electrophoresis, as described under "Experimental Procedures." The values shown are the average ones for the slices from the duplicates. The values for the total radioactivity in the Glut 1 peak of the duplicates were: basal, 71 and 100 cpmp; insulin, 668 and 600 cpmp, and in the Glut 4 peak, basal, 29 and 37 cpmp; insulin, 355 and 386 cpmp. Each lane contained 45% of the immunoprecipitate from the 35-mm plate. The numbers at the top show the molecular mass markers in kilodaltons.

Fig. 3. Labeling of Glut 1 and 4 with ATB-BMPA. Duplicate 35-mm plates of basal (C) and insulin-treated 3T3-L1 adipocytes (O) were labeled with [3H]ATB-BMPA and Glut 1 and 4 were isolated by immunoprecipitation and gel electrophoresis, as described under "Experimental Procedures." The values shown are the average ones for the slices from the duplicates. The values for the total radioactivity in the Glut 1 peak of the duplicates were: basal, 71 and 100 cpmp; insulin, 668 and 600 cpmp, and in the Glut 4 peak, basal, 29 and 37 cpmp; insulin, 355 and 386 cpmp. Each lane contained 45% of the immunoprecipitate from the 35-mm plate. The numbers at the top show the molecular mass markers in kilodaltons.

Interpretation of the increase in ATB-BMPA labeling in response to insulin as due to translocation involves the assumption that insulin does not alter the intrinsic reactivity of the transporter at the cell surface with this reagent (see
The entire experiment shown in Fig. 4 gave the same results.

The fractions show a strong peak of Glut 1 in human erythrocyte membranes at 20 °C (26).

The data is adequately fit by the assumption that inhibition is due to binding to a single site, with a dissociation constant of 530 PM in basal cells and 420 PM in insulin-treated cells. Thus, insulin does not significantly alter the apparent affinity of the transporters for ATB-BMPA. This value for the dissociation constant is similar to the value of 300 PM found for the binding of ATB-BMPA to Glut 1 in human erythrocyte membranes at 20 °C (26).

It might have been expected that Glut 1 and Glut 4 would have different affinities for ATB-BMPA, and therefore that the plots in Fig. 3 would be curved. Two possible explanations for the linear plots are that Glut 1 and 4 have very similar affinities for ATB-BMPA and/or that the contribution of one to the transport rate is a relatively small fraction of the total.

### Table I

| Experiment | Basal | Glut 1 insulin | -Fold* | Basal | Glut 4 insulin | -Fold* | dGlucose uptake -fold |
|------------|-------|----------------|--------|-------|----------------|--------|-----------------------|
| 1          | 72, 100 | 548, 600 | 6.7    | 29, 37 | 355, 386 | 11.2   | 36                    |
| 2          | 53, 74  | 403, 415 | 6.3    | 29, 33 | 431, 396 | 11.9   | 27                    |
| 3          | 84, 91  | 400    | 4.6    | 6, 10 | 194, 265 | 28.0   | 12                    |
| 4          | 66, 75  | 442, 491 | 6.6    | 18, 23 | 244, 216 | 11.0   | ND                    |
| 5          | 34, 43  | 300, 330 | 6.2    | 6, 13 | 212, 229 | 23.5   | 10                    |
| Average    |        |              | 6.5    |        |              | 17.1   | 21                    |

* Ratio of the value for insulin-treated cells to that for basal cells.

* Only a single determination for Glut 1 insulin in this experiment.

Colocalization of Glut 1 and 4 in Vesicles

Previously, we have described a method for the isolation of the intracellular vesicles containing Glut 1 from the bulk of membranes in the 16,000 × g supernatant from 3T3-L1 adipocytes (10). This consists of immunoadsorption of these vesicles onto Staph A cells coated with antibodies against the carboxyl-terminal peptide of Glut 1. The procedure is quite selective, since only 10 μg of membrane (as protein) out of the 300 μg of membranes present in the 16,000 × g supernatant are adsorbed. In order to determine whether Glut 4 is located in the same vesicles as Glut 1, we immunoabsorbed the Glut 1-containing vesicles and then immunoblotted both the unadsorbed membranes and the adsorbed ones for Glut 4 (as well as for Glut 1). Since it seemed possible that even if all the Glut 4 were located in vesicles containing Glut 1, these might only constitute a fraction of the Glut 1-containing vesicles, the supplementary experiment in which membranes were adsorbed with antibodies against the carboxyl-terminal peptide of Glut 4 on Staph A cells was also performed.

The results of one experiment of this type are presented in Fig. 6. As expected, the antibodies against the carboxyl terminus of Glut 1 adsorbed the membranes containing Glut 1 (part A, lanes 5–7), whereas control antibodies did not (part A, lanes 2–4). Virtually all the Glut 4 was associated with the vesicles containing Glut 1 (part B, lanes 5–7). Similarly, as expected, the antibodies against the carboxyl terminus of Glut 4 adsorbed the membranes containing Glut 4 (part B, lanes 8–10), whereas control antibodies did not (part B, lanes 2–4). Most of the Glut 1 was associated with vesicles also containing Glut 4 (part A, lanes 8–10). In nine experiments of this type in which the adsorption of Glut 4 by Staph A cells coated with the antibodies against Glut 1 was determined, the extent of adsorption of Glut 4 averaged 95% of the total in the 16,000 × g supernatant. In four experiments where the adsorption of Glut 1 by Staph A cells coated with the antibodies against Glut 4 was examined, the degree of adsorption of Glut 1 averaged 85% of the total.

The morphology of the membranes containing Glut 1 and 4 isolated by immunoadsorption on the Staph A cells was examined by electron microscopy. A typical field is shown in Fig. 7A. The transporter-containing membranes were vesicles of heterogeneous size. The 20 vesicles in this field ranged from 35 to 196 nm in diameters, with the average value of 72 nm. The range and average were about the same for six other
 lines express Glut 1, but not Glut 4, whereas Glut 4 is present in rat heart and skeletal muscle at three to four times the level of Glut 1 (24).

In an effort to measure the increase in the amounts of the two transporters at the cell surface in response to insulin, we employed the membrane-impermeant, photoactivated affinity labeling reagent, ATB-BMPA. The use of this reagent involves the assumption that the intrinsic reactivity of each transporter with the reagent is not altered by insulin. The finding that insulin does not change the apparent affinity of the transporters for the reagent, as measured by its $K_v$ value for inhibition of transport, supports this assumption, but does not prove it. Nevertheless, it seems likely that increases in ATB-BMPA labeling reflect increases in the amounts of the transporter at the cell surface. The results show that the -fold increase in Glut 4 (average 17-fold) was considerably greater than the -fold increase in Glut 1 (average 6-fold). These results are similar to those found upon labeling rat adipocytes with ATB-BMPA, where insulin increased the label in Glut 1 and 4 by 5- and 20-fold respectively.2

The redistribution of the two transporters from the intracellular to the plasma membrane-containing fractions of the sucrose gradient in response to insulin provides further evidence for the translocation of each transporter. The fact that the -fold increases in the plasma membrane-containing fractions are considerably less than those found by the ATB-BMPA labeling method (2- and 4-fold, respectively, for Glut 1 and 4) may be due to the contamination of these fractions with intracellular membranes containing the transporters. Immunoelectron microscopic studies suggest that the translocatable intracellular Glut 1 and 4 are located in the trans-Golgi network and possibly also the endosomes (see below). However, there is no known marker for the intracellular membranes containing the insulin-responsive transporters that can be used to assess the contamination of the plasma membrane fractions (10). In a recent study with rat adipocytes, the plasma membrane fraction was isolated from basal and insulin-treated cells and analyzed for its Glut 1 and 4 contents by immunoblotting (20). Insulin treatment increased Glut 1 by 1.6-fold and Glut 4 by 8-fold.

The question of whether the entire increase in the rate of hexose transport in response to insulin is due to translocation of Glut 1 and 4 to the plasma membrane involves the following considerations. With the concentration of 2-deoxyglucose ([S]) much less than its half-saturation constant, the basal and insulin stimulated rates of transport ($v_b$ and $v_i$) are described by:

$$
v_b = \frac{k_1}{K_a} (\text{Glut 1}_b) + \frac{k_4}{K_a} (\text{Glut 4}_b) [S]
$$

$$
v_i = \frac{k_1}{K_a} (\text{Glut 1}_i) + \frac{k_4}{K_a} (\text{Glut 4}_i) [S]
$$

where $k_1$ and $k_4$ are the turnover numbers for Glut 1 and Glut 4, respectively; $K_t1$ and $K_t4$ are the half-saturation constants; and (Glut 1) and (Glut 4) are the amounts of each transporter in the plasma membranes of basal and insulin-treated cells (subscript b and i). Thus, an answer to the question requires a knowledge of the relative amounts of Glut 1 and 4 in the plasma membrane in the basal and insulin state, as well as a knowledge of the relative values of $k_1/K_a$ and $k_4/K_a$. Note that this treatment assumes that $k_1/K_a$ and $k_4/K_a$ are not changed by insulin. If translocation does not account for the full effect of insulin, then increases in the intrinsic activity of each transporter (the $k/K_a$ parameter) must be considered.

Previous studies have shown that 3T3-L1 adipocytes contain both Glut 1 and 4 but did not determine the relative or absolute amounts of these two transporters (4-7). The results herein show that Glut 1 is the predominant transporter. Since there are about $2.1 \times 10^6$ cells/35-mm plate, the amounts of Glut 1 and 4 are about 950,000 and 280,000/cell. This ratio of the two transporters is in sharp contrast to that in rat adipocytes, where the ratio of Glut 1 to Glut 4, as determined by immunoblotting of total cell membranes, is about 0.08.2

This difference between the predominant transporter type found in the cultured cells and the tissue cells is not limited to adipocytes. We have recently found that three muscle cell

2D. M. Calderhead, E. M. Gibbs, and G. E. Lienhard, unpublished results.

3I. J. Kozka, A. E. Clark, and G. D. Holman, unpublished results.
FIG. 5. Distribution of Glu 1 and 4 in subcellular fractions. Homogenates of basal and insulin-treated 3T3-L1 adipocytes were fractionated on 15-37% sucrose gradients, and the fractions were analyzed for Glu 1 (left panels) and 4 (right panels) by immunoblotting (see "Experimental Procedures"). The lanes are P, pellets from sucrose gradient; 1-13, fractions numbered from the bottom of the gradient; H, homogenate. The SDS samples contained the following amounts (expressed as a percent of the total from a 10-cm plate of cells): Glu 1, Ps 0.40 and 0.20, fractions 1-13 0.47, Hs 0.10 and 0.05; Glu 4, Ps 1.6 and 0.8, fractions 1-13 0.95, Hs 0.40 and 0.20.

FIG. 6. Immunoadsorption of vesicles containing Glu 1 and 4. The 16,000 × g supernatant from a homogenate of basal 3T3-L1 adipocytes (6 ml) was incubated either with no addition (lane 1) or with Staph A cells coated with irrelevant rabbit IgG (lanes 2-4), with antibodies against the carboxyl-terminal peptide of Glu 1 (lanes 5-7), or with antibodies against the carboxyl-terminal peptide of Glu 4 (lanes 8-10). In each case the immunoadsorbent was at 6 μg of antibodies on 2 μl of Staph A cells/ml supernatant. After the immunoadsorption step, the membranes remaining unadsorbed were sedimented and dissolved in SDS sample buffer (lanes 1-3, 5, 6, 8, 9), and the vesicles adsorbed to the Staph A cells were released into the same volume of SDS sample buffer (lanes 4, 7, and 10). Part A shows the immunoblot for Glu 1; part B is for Glu 4. The loads/lane as a percent of the total from a 10-cm plate were part A, lanes 3, 6, 9 (3%); other lanes (6%); part B, lanes 3, 6, 9 (1.5%); other lanes (3%).

Our data do not contain the information needed to calculate a value for the ratio, vν/νμ, that can be compared with the ratio of the experimentally determined values, which ranged from 10- to 36-fold in various platings of the cells. However, one implication can be drawn. The average -fold increase in surface Glu 4 (17-fold) as assessed by ATB-BMPA labeling, approached that of the -fold stimulation of transport (21-fold), whereas the -fold increase in surface Glu 1 was considerably less (6-fold). Thus, if translocation is to account fully for the insulin effect, the kν/Kμ (Glu 4) terms must predominate in the equations for vν and vμ. But there are two reasons to expect that the amount of Glu 1 in the plasma membrane is greater than that of Glu 4 in both the basal and insulin state. First, on the assumption that the two transporters are equally susceptible to labeling with ATB-BMPA, the ratio of Glu 1 to 4 in the plasma membrane averaged 4.6 and 1.6 for the basal and insulin state, respectively (data in Table I). Second, on the basis of the distribution of the transporters between intracellular and plasma membrane fractions on the sucrose gradients and the total amounts of each, the ratio of Glu 1 to 4 in the plasma membrane is calculated to be 8.6 and 6.1 for the basal and insulin state, respectively. Thus, for
translocation to account for the full insulin effect, the intrinsic activity of Glut 4 ($k_a/K_m$) must be considerably larger than that of Glut 1 ($k_a/K_m$). 95% of the Glut 4 in the intracellular membranes of the 16,000 × g supernatant was present in vesicles that also contained Glut 1; conversely, 85% of the Glut 1 was present in vesicles that contained Glut 4. Consequently, these transporters were largely colocalized, with only a small fraction of the Glut 1 in separate vesicles. Previously, we have reported that Glut 1-containing vesicles in the 16,000 × g supernatant from basal cells on 35-mm plate contain about 2.5 and 2.4 μg of protein and phospholipid, respectively (10). Since 60% of the Glut 1 and 4 are in the 16,000 × g supernatant, it can be calculated that Glut 1 and 4 are 3.7 and 1.0% of the protein in these vesicles. Also, the number of transporters of each type in the average 72-nm vesicle can be roughly estimated from the amounts of the transporters in the 16,000 × g supernatant and the number of transporter-containing vesicles. The latter can be estimated from the amount of vesicle phospholipid and the expected molecular weight of the phospholipid in a 72-nm vesicle (see Ref. 10 for details). The values are 24 Glut 1 and 7 Glut 4 molecules/vesicle.

Our finding on the colocalization of intracellular Glut 1 and 4 contrasts with the results of a study with rat adipocytes where it was found that less than 10% of the Glut 1 is the vesicles isolated with antibodies against Glut 4 (20). Thus, 3T3-L1 adipocytes are less effective than rat adipocytes at intracellular segregation of the two types of transporters. Possibly this is because of the larger amount of Glut 1 in 3T3-L1 adipocytes. It should be noted that since as few as one molecule of transporter/vesicle may be required for immunoadsorption of a vesicle, it is not possible to decide whether the transporter-containing vesicles in 3T3-L1 adipocytes are heterogeneous in the sense that some contain a much higher ratio of Glut 1 to 4 than others. The best approach to examine this issue will be to determine simultaneously the cellular distribution of both transporters in basal and insulin-treated cells by immunocytochemistry on cell sections, through detection with protein A-gold particles of two sizes. Previously, it has been demonstrated by immunoelectron microscopy that the insulin-responsive intracellular pool of Glut 1 is located in tubulovesicles in the trans-Golgi region of the 3T3-L1 adipocytes (8). A recent similar study with brown fat tissue has shown that the insulin-responsive Glut 4 is also located in the trans-Golgi area, as well as near large endocytic vacuoles, and preliminary results with basal 3T3-L1 adipocytes show concentration of Glut 4 in the trans-Golgi network.

In conclusion, the two types of glucose transporters in 3T3-L1 adipocytes are qualitatively similar in their response to insulin and in their subcellular distribution. However, the fold increase in Glut 4 at the cell surface in response to insulin is several times larger than that of Glut 1. The subcellular fractionation data suggest that the basis of this difference may lie in a greater tendency of Glut 4 to be located intracellularly in the basal state. The two types of transporters differ in 37% of their amino acid sequences (5). In the future it may be possible to identify particular sequences accounting for differences in insulin responsiveness through expression of chimaeric transporters in 3T3-L1 adipocytes (25). Finally, we note that mRNA encoding a third isoform of glucose transporter (Glut 3) has been detected in human fat tissue (27). When antibodies to this form become available it will be of interest to determine whether Glut 3 is also expressed in 3T3-L1 adipocytes and, if so, whether it translocates in response to insulin.

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