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Immunoelectron microscopic demonstration of insulin-stimulated translocation of glucose transporters to the plasma membrane of isolated rat adipocytes and masking of the carboxyl-terminal epitope of intracellular GLUT4

(glucose transport/insulin action/epitope masking)

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ABSTRACT **Polyclonal antibodies to the amino- or carboxyl-terminal peptide sequences of the GLUT4 transporter protein were used in immunoelectron microscopic studies to demonstrate the location and insulin-induced translocation of GLUT4 in intact isolated rat adipocytes. Labeling of untreated adipocytes with the amino-terminal antibody revealed 95% of GLUT4 was intracellular, associated with plasma membrane invaginations or vesicles contiguous with or within 75 nm of the cell membrane. Insulin treatment increased plasma membrane labeling ≈13-fold, to 52% of the total transporters, and decreased intracellular labeling proportionately. In contrast, labeling of untreated adipocytes with the carboxyl-terminal antibody or with a monoclonal antibody (1F8) that binds to the carboxyl terminus of GLUT4 detected fewer transporters, only ≈40% of which were intracellular. In insulin-treated cells, plasma membrane labeling increased ≈20-fold, but the total number of labeled transporters also increased ≈13-fold. The number of intracellular transporters was not changed. The insulin-induced increase in plasma membrane labeling was reversible. Thus, the vast majority of GLUT4 transporters in untreated adipocytes are intracellular in invaginations or vesicles attached or close to the plasma membrane. Insulin treatment causes translocation of transporters to the plasma membrane, which involves flow of transporters from invaginations to the cell surface and possible fusion of subplasma membrane vesicles with the plasma membrane. Differences in the labeling of intracellular transporters by peptide antibodies suggested the carboxyl-terminal epitope of intracellular transporters was masked. The unmasking of the carboxyl terminus during translocation to the plasma membrane may be part of the mechanism by which insulin stimulates glucose transport in rat adipocytes.**

Insulin stimulates the rate of glucose transport in adipocytes up to 20-fold (1) by causing translocation of glucose transporters from an apparent intracellular compartment to the plasma membrane (2, 3). Insulin-induced translocation of glucose transporters also has been demonstrated in other cells or tissues (4–7). Debate continues over whether translocation is the sole mechanism involved in insulin-stimulated glucose transport or whether activation of transporters in the plasma membrane (8–12) also occurs.

Immunoelectron microscopy (IEM) has been used to localize glucose transporters in 3T3-L1 adipocytes (13), muscle tissue (14, 15), brown adipose tissue (15, 16), endothelial tissue (17), and other cells. Differences in results do not allow general conclusions to be drawn as to the location of intracellular transporters or the manner by which translocation occurs. The present study is an IEM characterization of glucose transporters in isolated rat white adipocytes, a cell type used extensively in biochemical studies. The structure of the white adipocyte has presented numerous problems in preparing these cells for electron microscopy. We developed methods for preparing adipocytes for electron microscopy to study the processing of the insulin receptor (18). Here, those methods were modified to retain antigenicity of the glucose transporter.

GLUT4 is the predominant glucose transporter isoform in white adipose cells as well as in other cells in which glucose transport is stimulated by insulin. In unstimulated adipocytes GLUT4 is localized to presumably intracellular structures that have been called "low density microsomes" based on cell fractionation techniques. Here we show that in untreated cells the vast majority of GLUT4, detected with an amino-terminal peptide antibody, was intracellular and associated with plasma membrane invaginations, surface-connected vesicles (18), or vesicles within 75 nm of the plasma membrane. Thus, the low density microsomes are closely associated with the plasma membrane. Insulin treatment caused a 13- to 20-fold increase in the labeling of plasma membrane GLUT4 proteins that was reversed when insulin-treated cells were washed and reincubated in the absence of insulin. Despite similar labeling by amino- and carboxyl-terminal antibodies of plasma membrane GLUT4, labeling of intracellular transporters in untreated adipocytes by the carboxyl-terminal peptide antibody and a monoclonal antibody (1F8) that binds to the carboxyl terminus was markedly reduced. These observations suggest the intracellular carboxyl-terminal epitope was masked. Epitope masking might be caused by differences in the conformation of the carboxyl terminus of the transporter protein or differences in the protein or lipid surrounding the transporter. Since the carboxyl-terminal epitope is unmasked in the plasma membrane, changes leading to the unmasking, along with the translocation of the transporter, may be part of the mechanism by which insulin stimulates glucose transport in adipocytes.

MATERIALS AND METHODS

Cell Isolation and Incubation. Isolated rat adipocytes were prepared from the epididymal fat pads of 100-g male Sprague–Dawley rats as described (19). The cells were washed three

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Abbreviation: IEM, immunoelectron microscopy.

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times and resuspended in Krebs/Ringer Mops buffer at pH 7.4 [KRM buffer: 128 mM NaCl/5 mM KCl/1.5 mM $MgSO_4$/5 mM NaH_2PO_4/1.5 mM $CaCl_2$/25 mM 3-(*N*-morpholino)propanesulfonic acid (Mops)] containing 3% (wt/vol) bovine serum albumin and 2 mM glucose. The cells were kept at 37°C at all times during the isolation procedure. Adipocytes ($\approx 5 \times 10^5$ per ml) were incubated in the absence or presence of insulin (10 ng/ml; 1.7 nM) for 30 min at 37°C or as indicated. In each experiment, one aliquot of insulin-treated cells was washed three times at 37°C in insulin-free KRM buffer, resuspended in KRM buffer, and incubated an additional 30 min in the absence of insulin.

IEM. Cells were centrifuged at 500 × *g* for 30 sec and the infranatant beneath the floating cells was removed. The cells were fixed in a large volume of IEM fixative (4% paraformaldehyde/50 mM $K_3Fe(CN)_6$/1 mM $CaCl_2$ in 0.1 M sodium cacodylate, pH 7.4) for 60 min at 24°C. Fixed cells were washed three times with 0.1 M sodium cacodylate and embedded in 2% agar as described (18). Cubes (≈ 1 mm^3) were cut from the solidified agar. The specimens were dehydrated with ethanol (50–90%) and embedded in LR White resin. Thin sections ($\approx$700 Å) were cut and collected on nickel grids. Within 4 hr, the sections were incubated for 60 min at 24°C in wetting/blocking solution [1% ovalbumin in phosphate-buffered saline (PBS) at pH 7.4] followed by 50 μg of antitransporter IgG (see below) or control IgG per ml for 48 hr at 4°C in a humidified chamber. IgG fractions were prepared by ammonium sulfate precipitation (20) from serum, dialyzed against PBS, and stored at 0°C. IgG fractions were used within 48 hr of purification. The sections were washed four times with 1% ovalbumin in 10 mM sodium phosphate buffer at pH 8.0 and then incubated for 60 min with gold-labeled protein A. The sections were washed four times with 10 mM Tris·HCl-buffered normal saline and deionized water prior to staining for 5 min in neutralized 2% aqueous uranyl acetate. Control conditions included substitution of equal concentrations of normal rabbit IgG or preimmune IgG for immune IgG. In addition, peptide IgGs MC1A and MC2A were preincubated for 2 hr with a 10-fold molar concentration of peptide used to prepare the antibodies. $IgG_{(n)}$:peptide aggregates were removed by Microfuge centrifugation.

Quantitative Morphometric Analysis. Data reported are results of three separate cell preparations and incubations. Thin sections were cut from three or more randomly selected tissue samples for each experimental condition in each experiment. At least 25 different thin-sectioned cells were examined in each experiment for each condition. The total number of gold particles per thin-sectioned cell profile was determined as well as the number of particles associated with the plasma membrane and various intracellular organelles, including plasma membrane invaginations, subplasma membrane and cytoplasmic vesicles, endosomes, lysosomes, mitochondria, and nuclei. In previous studies we have shown that some of subplasma membrane and cytoplasmic vesicles are connected to the plasma membrane at a depth above or below the plane of section (18, 21). Although there was some cell-to-cell variation in cell diameter and depth of cytoplasm, when pooled data of 25 cells were examined no significant differences in cell diameter or depth of cytoplasm were detected between experimental conditions or different experiments (data not shown).

Nonspecific binding of MC1A and MC2A antibodies was considered negligible (see *Results*) and no corrections were made for nonspecific binding. Gold particles resulting from 1F8 binding to nuclei, mitochondria, and other suspected spurious sites (see *Results*) were not included in the analysis of GLUT4 localization. Results expressed are the means ± SD of the observations in three experiments; significance values were determined with a two-tailed *t* test.

Antibodies. Peptides containing the amino-terminal 13 amino acids (CMPSGFQQIGSEDG) and the carboxyl-terminal 16 amino acids (CVKPSTELEYLGPDEND) of the rat GLUT4 protein were synthesized (Multiple Peptide Systems, San Diego) and coupled to keyhole limpet hemocyanin as described by Green *et al.* (22). Rabbits were immunized, and antibodies were obtained and characterized as described by Kahn *et al.* (23). These antibodies are identified as MC1A (amino-terminal peptide) and MC2A (carboxyl-terminal peptide). Mouse anti-rat "insulin-regulated" glucose transporter monoclonal antibody, identical to antibody 1F8 (24), was purchased from Genzyme (product code 1262-00).

RESULTS

Fig. 1 *A–F* demonstrate the localization of GLUT4 proteins in rat adipocytes detected with MC1A amino-terminal peptide antibody. In untreated adipocytes (Fig. 1 *A* and *B*) the majority of the transporters were found in invaginations, surface connected vesicles (18, 21), or vesicles within $\approx$75 nm of the plasma membrane. The distribution of labeled structures and the relative intensity of their labeling were random and uniform around the entire perimeter of the cell. Insulin treatment (Fig. 1 *C* and *D*) caused a marked increase in the labeling of the plasma membrane surface with an apparently concomitant decrease in the labeling of membrane invaginations, surface-connected vesicles, and subplasma membrane vesicles. Increased plasma membrane labeling was seen as early as 5 min after insulin addition and labeling was random and uniform along the entire circumference of the cell between 5 and 180 min after insulin addition (data not shown). Insulin treatment caused no apparent change in the number of subplasma membrane vesicles or membrane invaginations (data not shown). When insulin-treated adipocytes were washed and incubated for an additional 30 min in the absence of insulin, the distribution of GLUT4 proteins was virtually identical to that observed in untreated cells (Fig. 1*E*).

Preincubation of MC1A antibody with a 10-fold molar excess of amino-terminal peptide reduced binding of MC1A to negligible levels in insulin-treated cells (Fig. 1*F*). Similar results were observed with untreated or insulin-treated and washed cells (data not shown). Quantitative analysis revealed that peptide preincubation reduced the number of observed gold particles in all incubation conditions by 89.2% ± 2.1%. In contrast, preincubation of MC1A with the carboxyl-terminal peptide had no effect on the number of gold particles observed (102.3% ± 3.2% of control).

As shown in Fig. 2*A*, MC2A antibody, specific for the carboxyl-terminal peptide of GLUT4, detected small amounts of the transporter in vesicular structures in the cytoplasm and on the plasma membrane of untreated adipocytes. Insulin-treated cells (Fig. 2*B*) showed a marked increase in labeling of the plasma membrane, although no apparent change was seen in the labeling of intracellular structures. When insulin-treated adipocytes were washed and incubated in the absence of insulin, the increased labeling of the cell membrane was reversed, although no change in intracellular labeling was observed compared to either untreated or insulin-treated cells (not shown). Preincubation of MC2A antibody with a 10-fold molar excess of carboxyl-terminal peptide reduced the number of gold particles in all incubation conditions by 96.8% ± 2.1%. Preincubation of MC2A with the amino-terminal peptide had no effect on the number of gold particles observed (96.8% ± 3.4% of control).

Other IEM studies (14, 15, 17) have used a monoclonal anti-glucose transporter antibody, 1F8 (24). We used this antibody to compare its localization of glucose transporters to that observed with MC2A because 1F8 it also binds to the carboxyl terminus of GLUT4 (15). Significant amounts of

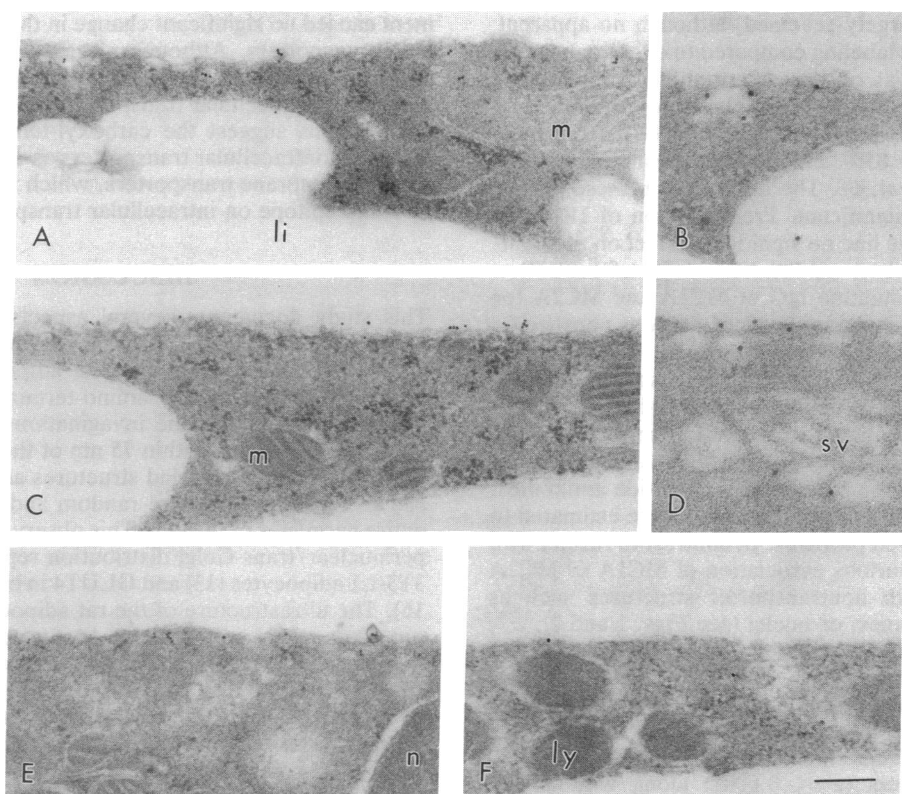

FIG. 1. Immunostaining of isolated rat adipocytes with amino-terminal peptide antibody. Adipocytes were incubated in the absence or presence of 10 ng of insulin per ml for 30 min at 37°C. One aliquot of insulin-treated cells was washed and reincubated for 30 min in the absence of insulin. The cells were prepared for IEM and immunostained with MC1A, the polyclonal antibody to the amino-terminal peptide of GLUT4. Antibody binding sites were detected with gold-labeled protein A. (*A* and *B*) Untreated adipocytes. (*C* and *D*) Insulin-treated adipocytes. (*E*) Insulin-treated adipocytes, washed and reincubated without insulin. (*F*) Insulin-treated adipocytes immunostained with MC1A that had been preincubated with a 10-fold molar excess of peptide. li, Central lipid droplet; ly, lysosome; m, mitochondria; n, nucleus; sv, surface-connected vesicles. (Bar = 0.2 μm for *A*, *C*, *E*, and *F* or 0.13 μm for *B* and *D*.)

"spurious" binding were detected over lysosomes, mitochondria, and nuclei (Fig. 3 *A* and *B*), as previously reported (15), and accounted for 56% of the intracellular particles. In addition, other gold particles in the cytoplasm did not appear to be associated with any structures. Despite these artifacts, insulin-treated cells (Fig. 3*B*) had more gold particles on the plasma membrane than did untreated cells (Fig. 3*A*). When adipocytes were incubated with insulin, washed, and incubated in the absence of insulin, the increased labeling of the

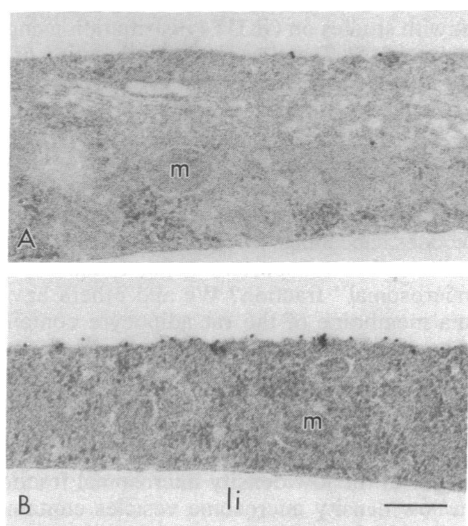

FIG. 2. Immunostaining of isolated rat adipocytes with carboxyl-terminal peptide antibody. The conditions were as described for Fig. 1 except the thin-sectioned cells were immunostained with MC2A, the polyclonal antibody to the carboxyl-terminal peptide of GLUT4. (*A*) Untreated adipocytes. (*B*) Insulin-treated adipocytes. li, Central lipid droplet; m, mitochondria; n, nucleus. (Bar = 0.2 μm.)

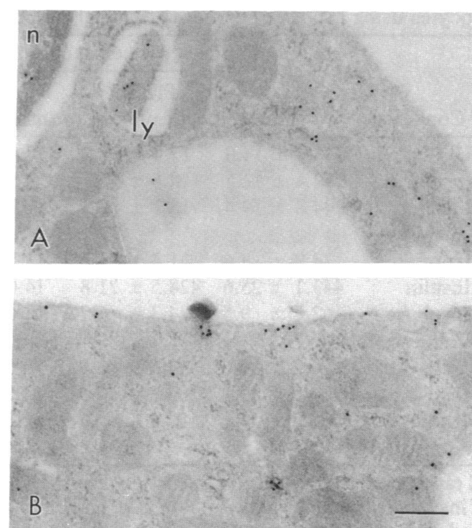

FIG. 3. Immunostaining of isolated rat adipocytes with 1F8 antibody. The conditions were as described for Fig. 1 except the thin-sectioned cells were immunostained with 1F8, the monoclonal antibody to GLUT4 (23). (*A*) Untreated adipocytes. (*B*) Insulin-treated adipocytes. ly, Lysosome; n, nucleus. (Bar = 0.2 μm.)

cell membrane was largely reversed, although no apparent change in intracellular labeling compared to either control or insulin-treated cells was observed (not shown). Preincubation of 1F8 antibody with a 10-fold molar excess of carboxyl-terminal peptide used to prepare MC2A reduced labeling over spurious locations by 85% ± 6.2% and presumed specific locations by 95.4% ± 4.3%. The difference in these results was not statistically significant. Preincubation of 1F8 with amino-terminal peptide had no significant effect on the number of gold particles observed (88.4% ± 6.7% of control).

Substitution of preimmune IgG of MC1A and MC2A for peptide IgG resulted in a low level of random nonspecific labeling that was less than that observed in the peptide preincubation controls (not shown). These results, along with the significant ($P < 0.001$) inhibition observed when peptide antibodies were preincubated with a 10-fold molar excess of specific peptide, suggest the gold-protein A particles observed represent specific binding of the peptide antibodies. Nonspecific cell-associated gold particles were estimated to be <5% of the observed particles. In contrast to results with 1F8, there was no spurious association of MC1A or MC2A immunoglobulins with nontransporter structures such as mitochondria, lysosomes, or nuclei (see Figs. 1 and 2).

Table 1 shows the mean total number of transporters detected per thin-sectioned cell with MC1A amino-terminal peptide antibody was not significantly different in untreated, insulin-treated, or insulin-treated and washed cells. Insulin treatment caused a 13-fold increase in the number of plasma membrane transporters ($P < 0.0001$) along with a 41% reduction in intracellular labeling ($P < 0.001$) detected with MC1A. Intracellular labeling included gold particles in plasma membrane invaginations, surface-connected vesicles, or subplasma membrane vesicles. Plasma membrane labeling included only plasma membrane surface gold particles. Reincubation of insulin-treated cells in the absence of insulin for 30 min returned the GLUT4 proteins to the distribution observed in untreated cells.

Both carboxyl-terminal antibodies, MC2A and 1F8, gave different results. Insulin treatment increased the total number of detected transporters by 4-fold (1F8) to 13-fold (MC2A) (both at $P < 0.001$). Virtually all of this increase was caused by an increase in plasma membrane labeling. Insulin treatment caused no significant change in the labeling of intracellular transporters. Although plasma membrane labeling in insulin-treated and washed cells returned to control levels, labeling of intracellular transporters was not affected. These observations suggest the carboxyl-terminal antibodies did not detect intracellular transporters as effectively as they did plasma membrane transporters, which implies the carboxyl-terminal epitope on intracellular transporters is masked.

Table 1. IEM detection of GLUT4 glucose transporters in intact white adipocytes using amino- or carboxyl-terminal antibodies

		Particles per cell section		
Antibody	Condition	Total	Plasma membrane	Intracellular
MC1A	Untreated	246.8 ± 14.8	11.4 ± 1.2	235.4 ± 17.8
	Insulin	287.4 ± 12.5	148.6 ± 13.7	138.8 ± 11.5
	Insulin and wash	244.8 ± 19.4	15.2 ± 3.4	241.9 ± 18.7
MC2A	Untreated	34.9 ± 3.6	21.4 ± 2.4	13.5 ± 1.8
	Insulin	443.1 ± 25.6	428.5 ± 21.8	14.6 ± 2.4
	Insulin and wash	62.8 ± 9.5	44.3 ± 6.7	18.6 ± 3.6
1F8	Untreated	74.2 ± 10.5	14.6 ± 1.9	59.6 ± 6.8
	Insulin	300.0 ± 33.7	235.8 ± 17.4	64.2 ± 8.4
	Insulin and wash	78.9 ± 11.6	20.5 ± 4.5	58.3 ± 6.6

Isolated rat adipocytes were incubated in the absence or presence of 10 ng of insulin per ml for 30 min. An aliquot of insulin-treated cells was washed and reincubated in the absence of insulin for 30 min. Cells were prepared and stained for IEM. Quantitative analysis was performed on 25 thin-sectioned adipocytes in each of three different experiments for each condition. Data are the mean ± SD. Intracellular gold particles over nuclei, mitochondria, and lysosomes were not included in the analysis of 1F8 distribution (see *Results*). The number of particles counted ranged from 800 to 11,000 per condition.

DISCUSSION

This study documents several aspects of the intracellular localization of GLUT4 in basal and insulin-stimulated rat white adipocytes. In untreated adipocytes, 95% of the GLUT4 detected by the amino-terminal peptide antibody MC1A was in membrane invaginations, surface-connected vesicles, or vesicles within 75 nm of the plasma membrane. The distribution of labeled structures and the relative intensity of their labeling were random and uniform around the entire perimeter of the cell. This observation differs from the perinuclear/trans-Golgi distribution reported for GLUT1 in 3T3-L1 adipocytes (13) and GLUT4 in brown adipocytes (15, 16). The ultrastructure of the rat adipocyte is unique and is not similar to that of 3T3-L1 or brown adipocytes. Cells used in this study were 90–120 μm in diameter, most of which was the lipid droplet. The cytoplasm was a thin layer, 0.5–2 μm deep, surrounding the central lipid droplet. The thickest portion of the cytoplasm is around the nucleus, where the highest concentration of Golgi is located (25–27). This area of the cell lies under <5% of the plasma membrane. The density of GLUT4 in the perinuclear area was similar to that in other regions of the cytoplasm under all incubation conditions. The rapid translocation of GLUT4 to the plasma membrane and the uniform distribution of GLUT4 along the membrane of insulin-treated adipocytes suggest a dispersed intracellular store of GLUT4 may be more efficient than concentrating the transporter in the perinuclear region.

In insulin-treated adipocytes, 52% of the GLUT4 detected by the amino-terminal peptide antibody was associated with the plasma membrane, a 13-fold increase in the number of transporters compared to untreated cells. There was a corresponding decrease in the labeling of intracellular structures. Immunostaining of insulin-treated and washed cells demonstrated these effects were reversible. These results are consistent with studies on GLUT4 distribution using subcellular fractionation (2–7) and suggest translocation of GLUT4 proteins to the plasma membrane is the predominant mechanism by which insulin activates glucose transport in these cells.

The location of GLUT4 in invaginations, surface-connected vesicles, and subplasma membrane vesicles in untreated rat adipocytes raises several questions.

(*i*) How does subcellular fractionation of untreated adipocytes result in the transporters being found in the "low density microsomal" fraction? We and others have shown the plasma membrane of the rat adipocyte contains many invaginations that often form multiple vesicle-shaped buds that are connected to the cell surface (18, 21, 27), as shown in Fig. 1 *A*, *B*, and *D*. It is likely that these invaginations and surface-connected vesicles are sheared from the plasma membrane during homogenization and form small vesicles that sediment in the low density microsomal fraction. Only 3% of the low density microsome vesicles contain glucose transporters (28). Furthermore, attempts to characterize the vesicles based on marker enzymes generally have been unsuccessful (2–4, 29). These results suggest the vesicle containing the glucose transporter is a unique structure and, although it may sediment in the low density microsomes with vesicles of similar size and density from other cellular organelles, it is probably not part of the endoplasmic reticulum,

Golgi apparatus, endosomal and lysosomal compartments, or the plasma membrane *per se*. Based on their structural appearance in the isolated adipocyte, invaginations and surface connected vesicles may have unique chemical compositions making them biochemically distinct from other cellular organelles.

(*ii*) How does intracellular GLUT4 move to the cell surface? Transporters located in invaginations and surface-connected vesicles could flow in the plane of the membrane to the cell surface. Supporting this hypothesis is a report showing *cis*-vaccenic acid, an agent that increases membrane fluidity, stimulated glucose transport comparably to insulin, whereas stearate, which decreases membrane fluidity, decreased insulin-stimulated glucose transport (30). A physical translocation, involving vesicle fusion, would be necessary for the movement of transporters from non-surface-connected vesicles to the cell surface. The mechanism by which insulin might cause either form of translocation is unknown. However, insulin affects the membrane/intracellular distribution of other proteins (31–33), including its own receptor (34).

(*iii*) If GLUT4 transporters are in invaginations and surface-connected vesicles, why do they need to be translocated to the cell surface? One might presume that transporters in those structures are exposed to the extracellular environment, which would include glucose or photoaffinity labels (7) used to quantify plasma membrane-associated transporters. Fixation and staining techniques compatible with IEM in this study did not reveal any obstruction in the neck of the invaginations. However, in previous studies we and others have described electron-opaque material within and at the opening of the invaginations (21, 26). It is possible that this unidentified material may present a barrier to glucose and other extracellular components (27).

On the basis of the results with both carboxyl-terminal antibodies (MC2A and 1F8), we propose that the carboxyl-terminal epitope of GLUT4 is masked in its intracellular location. Epitope masking of intracellular transporters is not evident in biochemical studies utilizing Western blotting of solubilized transporters or immunoprecipitation of transporter vesicles from the low density microsome fraction. However, there is no reason to believe the native conformation or milieu of the transporter has been maintained during the procedures preceding the use of these techniques. Our IEM results suggest the carboxyl-terminal GLUT4 epitope was unmasked when insulin triggered translocation of intracellular GLUT4 to the plasma membrane surface. Alternatively, insulin treatment may cause unmasking, which then results in translocation. "Unmasking" of the carboxyl terminus of GLUT4 protein could result from a change in orientation or conformation of the transporter or a change in the lipids and proteins surrounding the transporter. This unmasking process, combined with translocation of the transporters, may be part of the mechanism by which insulin stimulates glucose transport in adipocytes.

Other IEM studies have used similar carboxyl-terminal peptide (14–16) and 1F8 (14, 15, 17) antibodies. Our results agree with Slot *et al.'s* (15) assessment that 1F8 is not a suitable antibody for IEM studies because of its spurious reactions. The reported localization of GLUT4 in endothelial cells (17) detected with 1F8 has been contradicted (15). An IEM demonstration of insulin-induced translocation of GLUT4 was reported in muscle (14), but in preliminary observations in another study translocation in muscle was not as obvious as in brown adipocytes (15). Although the masking of the carboxyl terminus of intracellular GLUT4 proteins may be unique to the adipocyte, caution should be exercised when assessing the intracellular localization and translocation of GLUT4 in other tissues using 1F8 or other carboxyl-terminal transporter antibodies. Quantitative analysis, performed using labeling conditions where the ratio of gold particles to antigen remains relatively constant (35), should be used to determine whether or not epitope masking occurs. It is possible that epitope masking occurs not only with the glucose transporter but also with other cellular antigens—e.g., receptors, enzymes, G proteins—that redistribute following hormonal or other types of treatment.

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