Glucose deprivation enhances targeting of GLUT1 to lipid rafts in 3T3-L1 adipocytes

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Submitted 19 August 2003; accepted in final form 1 December 2003

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Glucose deprivation enhances targeting of GLUT1 to lipid rafts in 3T3-L1 adipocytes. Am J Physiol Endocrinol Metab 286: E568–E576, 2004. First published December 9, 2003; 10.1152/ajpendo.00372.2003.—Glucose deprivation dramatically increases glucose transport activity in 3T3-L1 adipocytes without changing the concentration of GLUT1 in the plasma membrane (PM). Recent data suggest that subcompartments within the PM, specifically lipid rafts, may sequester selected proteins and alter their activity. To evaluate this possibility, we examined the distribution of GLUT1 in Triton X-100-soluble and -insoluble fractions. Our data show that 77% of the GLUT1 pool in PMs isolated from control 3T3-L1 adipocytes was extracted by 0.2% Triton X-100. After glucose deprivation for 12 h, only 56% of GLUT1 was extracted by detergent. In contrast, there was a twofold increase in the GLUT1 content of the detergent-resistant fraction. To evaluate whether GLUT1 interacts with a specific protein within lipid rafts, we focused on stomatin, recently shown to interact with and inhibit GLUT1 activity. Stomatin is distributed about equally between the PM and the biosynthetic compartments, and its expression is not affected by glucose deprivation. Nearly 90% of the PM pool of stomatin is in detergent-resistant lipid rafts. In normal 3T3-L1 adipocytes, we were unable to demonstrate an interaction between GLUT1 and stomatin in coimmunoprecipitation experiments. However, in stomatin-overexpressing cells, there was clear coprecipitation of stomatin with GLUT1 antibodies. Glucose deprivation increased this interaction threefold, which may reflect the increase of GLUT1 in lipid rafts. Despite this, there was little change in transport activity in glucose-deprived, stomatin-overexpressing cells vs. that in control cells. Thus GLUT1 interacts with stomatin in lipid rafts, but this interaction per se does not alter transport activity. Rather, stomatin may serve as an anchor for GLUT1 in lipid rafts, the environment of which favors activation.

glucose transporter 1; lipid rafts; stomatin

MOST CELLS ARE DEPENDENT ON GLUCOSE UPTAKE and metabolism as a source of energy. Although the number of members of the transporter family is expanding, GLUT1 is ubiquitously expressed and is responsible for constitutive uptake in mammalian cells. The regulation of GLUT1 expression and activity has been intensively studied. Our focus has been on the nutrient-dependent regulation of glucose transport in 3T3-L1 adipocytes. Specifically, we (and others) have shown that a decrease in extracellular glucose concentration increases transport activity in 3T3-L1 adipocytes in a protein synthesis-dependent fashion (27, 39, 55, 56). Although complete glucose deprivation increases transport activity 10- to 15-fold, a change of fourfold is observed within the range of 2.5 to 25 mM, the limits of circulating glucose concentration in diabetic patients, suggesting physiological relevance. Although 3T3-L1 adipocytes express both GLUT1 and GLUT4, the insulin-stimulated transporter, the effect of glucose deprivation on transport activity in cultured cells is universal. Thus our attention has been drawn to the glucose-dependent regulation of GLUT1. Although complete glucose deprivation increases glucose uptake, as mentioned, it also induces aberrant protein glycosylation and the unfolded-protein response (UPR), which affects the expression of many proteins (26). To protect cells from the unwanted effects of glucose deprivation, we supplement glucose-deprived cells with fructose to prevent the UPR (26). Under these conditions, neither GLUT1 expression nor subcellular localization is affected, whereas elevated transport activity is maintained (12). From these data, we have hypothesized that GLUT1 is activated in response to glucose deprivation. One mechanism that could underlie activation is a new interaction between GLUT1 and a protein novel to glucose-deprived cells. Another possibility is the recruitment of GLUT1 from a nonfunctional to a functional domain within the plasma membrane (PM). In both scenarios, we envision GLUT1 in an inhibited state under glucose-fed conditions, one which is relieved by glucose deprivation. Recent studies from Zhang and colleagues (61, 62) showed that stomatin, a type II membrane protein originally identified in erythrocytes, interacts with and inhibits basal glucose uptake in Clone 9 cells. These derive from a liver cell line but express only GLUT1. Thus we thought it possible that GLUT1 was in an inhibited state in glucose-fed cells through its interaction with stomatin. Because stomatin has also been localized to detergent-resistant lipid rafts (28, 35), we thought that GLUT1 might be associated with this compartment by virtue of its interaction with stomatin. We thus tested these possibilities.

We now show that a subset of the GLUT1 pool in 3T3-L1 adipocytes resides in detergent-resistant lipid rafts and that glucose deprivation increases the GLUT1 content in these subdomains. This can be reversed by the readdition of glucose. Stomatin is localized to the PM and the membranes that comprise the biosynthetic compartments but not the intracellular pool that sequesters GLUT4. Stomatin expression is not affected by glucose deprivation. Within the PM, 90% of the stomatin pool resides in lipid rafts. GLUT1 specifically binds stomatin but only in stomatin-overexpressing cells. Interestingly, this interaction increases in glucose-deprived cells; yet overexpression of stomatin has little effect on glucose transport activity. Thus stomatin does not appear to act as a GLUT1 inhibitor in 3T3-L1 adipocytes. Rather, the association be-

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tween stomatin and GLUT1 may serve to target GLUT1 to lipid rafts. We propose that this redistribution of GLUT1 within the PM may favor activation rather than inhibition.

**MATERIALS AND METHODS**

*Materials.* Characterization of the polyclonal antibodies for GLUT1, GLUT4, and glucose-regulatory protein of 78 kDa (GRP78) have been previously published (26, 32). The Na+/H+ exchanger (NHE), and low-density membranes (LDM). Enrichment of specific fractions (49). Detergent extraction and isolation of lipid rafts. Normal 3T3-L1 adipocytes were washed three times with cold PBS and treated with 1.0 ml of buffer (10 mM HEPES, pH 7.5, 150 mM NaCl, 5 mM EDTA, and 0.5 mM PMFS) containing 1% Triton X-100 (TX-100) at 4°C by end-over-end rotation for 30 min. An aliquot was saved as the total extract (TE). Soluble and insoluble fractions were separated by centrifugation at 12,000 g for 30 min at 4°C, as described by Sakyo and Kitagawa (42).

For the isolation of lipid rafts, we used a slight modification of the protocol described by Chamberlain and Gould (6). Briefly, purified PM (1.0 mg) from glucose- and fructose-fed 3T3-L1 adipocytes were extracted in 1.0 ml of MBS buffer (25 mM MES and 150 mM NaCl, pH 6.5) containing 0.2% TX-100 and supplemented with protease inhibitor mix (Sigma). The samples were mixed end over end for 20 min at 4°C and then homogenized with 10 strokes of a Dounce homogenizer. These extracts were mixed with 1.0 ml of 80% sucrose. The samples (now in 40% sucrose) were placed at the bottom of centrifuge tubes and overlaid with 6 ml of 30% sucrose and 3.5 ml of 5% sucrose (in MBS). After centrifugation at 240,000 g in a Beckman SW-41 rotor for 18 h, 1.0-ml fractions were collected by upward displacement using 60% sucrose as the displacement fluid at 4°C. Protease inhibitor was added to each fraction. The fractions were mixed, and a 500-μl aliquot was treated with an equal volume of ice-cold 20% TCA. The samples were mixed and incubated at 4°C for 30 min. The precipitate was then dissolved in 0.1% SDS, and the proteins were resolved on a 12.5% SDS-PAGE gel under reducing conditions.

**Western blotting.** A rabbit antibody was generated against a stomatin peptide fragment, purified by affinity chromatography (Pierce), and used at a final concentration of 1 μg/ml. Details of the Western blotting procedure have been published previously (27).

**PM vesicle preparation and isolation with anti-stomatin antibodies.** PM vesicles of uniform size were prepared from isolated PM as described by Fisher and Frost (13). These vesicles were exposed to anti-stomatin antibodies bound to IgG-coated magnetic beads. A magnetic particle concentrator was used to collect immune complexes. The complexes were released with sample dilution buffer and processed for Western blotting.

**Immunoprecipitation.** 3T3-L1 adipocytes were extracted in PBS containing 2% polyoxyethylene 9 lauryl ether (C12E9) and 0.1% SDS, as described earlier (32). Extracts were subjected to centrifugation at 3,000 g for 5 min to remove insoluble protein complexes. The clarified extracts were exposed to preimmune serum to reduce nonspecific interactions before incubation with antibodies specific for either GLUT1 or stomatin. Immune complexes were collected using protein A-Sepharose washed with extraction buffer in the absence or presence of 1 M NaCl, as indicated in the legends for Figs. 1–8. The proteins were extracted in sample dilution buffer and processed for Western blotting.

**RESULTS**

Endogenous stomatin expression in 3T3-L1 adipocytes. Because the expression of stomatin in 3T3-L1 adipocytes has not been previously published (26, 32). The Na+/H+ exchanger (NHE), and low-density membranes (LDM). Enrichment of specific fractions (49). Detergent extraction and isolation of lipid rafts. Normal 3T3-L1 adipocytes were washed three times with cold PBS and treated with 1.0 ml of buffer (10 mM HEPES, pH 7.5, 150 mM NaCl, 5 mM EDTA, and 0.5 mM PMFS) containing 1% Triton X-100 (TX-100) at 4°C by end-over-end rotation for 30 min. An aliquot was saved as the total extract (TE). Soluble and insoluble fractions were separated by centrifugation at 12,000 g for 30 min at 4°C, as described by Sakyo and Kitagawa (42).

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been reported, we first sought to examine endogenous expression and to determine whether expression was affected by glucose deprivation (Fig. 1). We pretreated cells for 12 h in the presence of 25 mM glucose, glucose and chronic insulin (1 μM), and 25 mM fructose in the absence of glucose or in the complete absence of sugar. Total RNA was isolated for Northern blot analysis and probed with a mouse-specific stomatin cDNA probe (Fig. 1A). On the basis of our ability to detect stomatin mRNA by Northern blot analysis, we deduced that stomatin message must be relatively abundant. However, only complete hexose deprivation affected stomatin expression, decreasing mRNA by ~50%. Positive controls for this experiment included the upregulation of GLUT1 in the presence of chronic insulin treatment (14, 53) and the upregulation of GRP78 in the complete absence of sugar (26, 27). Indeed, insulin increased the expression of GLUT1, whereas complete hexose deprivation induced GRP78 expression. Note that cells exposed to fructose showed no change in GRP78 expression, indicating that fructose metabolism prevents the induction of the UPR.

To analyze protein expression, we isolated subcellular membrane fractions from 3T3-L1 adipocytes treated in the presence of glucose (+) or in the absence of glucose but the presence of fructose (−). Previously, we (12) have shown that 20% of the total GLUT1 pool is present in PM isolated from either glucose-fed or fructose-fed cells. The data in Fig. 1B confirm that GLUT1 expression does not change in response to glucose deprivation. GLUT1 was found in all three subcellular membrane compartments, the PM, the biosynthetic compartment (HDM), and the LDM fraction in which the GLUT4 translocatable pool resides. Based on densitometric analysis, the relative density of GLUT1 in the PM from glucose-fed cells in this experiment was 75 compared with 81 from fructose-fed cells. The relative density of GLUT1 in the PM was 201 for glucose-fed cells and 202 for fructose-fed cells. Note that there was essentially no GLUT4 in the PM in the presence or absence of glucose. GRP78, a protein resident to the ER, was enriched in the HDM fraction. As expected, Na⁺-K⁺-ATPase was found predominantly in the PM fraction. The stomatin peptide-specific antibody detected a 30-kDa protein that was localized to both the PM and HDM fractions. This pattern of expression is similar to that of the insulin receptor in this same cell line (19). Note that glucose deprivation (F) did not alter stomatin expression compared with controls (G). Together with the Northern blot analysis, these data show that stomatin is expressed in 3T3-L1 adipocytes and targeted to the PM. However, stomatin expression is not affected by glucose deprivation, suggesting that it is not the (or one among several) elusive protein synthesis-dependent factor underlying activated glucose uptake.

**Distribution of proteins within the PM.** Because stomatin has been localized to lipid rafts (43), we extracted cells in 1% TX-100 and separated the soluble from the insoluble fractions (Fig. 2). The TE contained an average of 5.09 ± 0.02 mg of protein in two independent experiments with no difference among the experimental conditions. The soluble fractions contained an average of 4.64 ± 0.02 mg of protein, and the insoluble fractions contained 0.48 ± 0.08 mg of protein. Because 70 μg were loaded onto the gels, a greater percentage of the insoluble fraction was loaded relative to the total extracts or soluble fraction. This accounts for the apparent low levels of

**Fig. 1.** Stomatin expression in 3T3-L1 adipocytes. A: cells were exposed to 25 mM glucose (G), 1 μM insulin in the presence of glucose (I), 25 mM fructose in the absence of glucose (F), or hexose-free medium (−H) for 12 h. Total RNA was collected, and 15 μg were separated on a 1% agarose gel. mRNA was probed for stomatin, GLUT1, and glucose-regulatory protein of 78 kDa (GRP78), as described in MATERIALS AND METHODS. Ethidium bromide staining is shown as an indication of loading. B: cells were exposed to 25 mM glucose (+) or 25 mM fructose in the absence of glucose (−H) for 12 h. Plasma membranes (PM), high-density membranes (HDM), and low-density membranes (LDM) were separated from fractionated cells. Proteins (50 μg) were separated by SDS-PAGE (12.5%) and analyzed for stomatin, Na⁺-K⁺-ATPase, GRP78, GLUT4, and GLUT1 expression by immunoblotting by use of a polyclonal antibody generated against specific sequences. Molecular mass markers in kDa are shown on the left.

**Fig. 2.** Distribution of GLUT1 and stomatin in Triton X-100 (TX-100)-extracted cells. 3T3-L1 adipocytes were exposed to 25 mM glucose or 25 mM fructose for 24 h or sequentially to fructose for 12 h and then glucose for 12 h. TfR1, transferrin receptor 1; TE, total extract; Sol, TX-100-soluble fraction; InSol, TX-100-insoluble fraction; G, glucose feeding; F, fructose feeding; F/G, fructose-to-glucose feeding. Cells were extracted in extraction buffer containing 1% TX-100 as described in MATERIALS AND METHODS. Insoluble material was collected by centrifugation. Equal protein was loaded onto a 12.5% SDS-PAGE gel. Proteins were electrophoretically transferred to nitrocellulose and analyzed by Western blot analysis.
stomatin in TE. Regardless, the data show that stomatin is found predominantly in the insoluble fractions, characteristic of proteins associated with lipid rafts. Once again, stomatin expression in this fraction did not change with fructose feeding, consistent with the aforementioned fractionation studies.

GLUT1 was also found in this insoluble fraction, but, remembering that the blot represents 15% of the entire insoluble fraction, we concluded that much of GLUT1 is extracted by TX-100, as noted by detection in the soluble fraction. Interestingly, fructose feeding increased the distribution of GLUT1 into the detergent-resistant fraction, with a consequent loss from the soluble fraction. This pattern could be reversed by the readdition of glucose. Similar to other reports in 3T3-L1 adipocytes, much of the caveolin was found in the detergent-resistant fraction. Again, glucose deprivation did not affect expression or distribution. TIR1, which is excluded from lipid rafts, was found only in the detergent-soluble fraction.

In contrast to whole cell extraction, recent studies suggest that extraction of isolated PM with 1% TX-100 completely disrupts lipid rafts (6). Thus we isolated PM and extracted them in the presence of 0.2% TX-100. We separated the detergent-resistant membranes by flotation on a 30% sucrose cushion (Fig. 3). The detergent-resistant fraction contained ~60% of the caveolin pool. Once again, glucose deprivation did not change this partitioning. Nearly 90% of the stomatin migrated with the detergent-resistant fraction and was not affected by glucose deprivation. However, the GLUT1 level in the soluble fraction decreased from 77 to 56% of the total PM pool. This corresponded to a twofold higher level of GLUT1 in the lipid raft fractions in fructose-treated cells compared with glucose-treated cells. Once again, TIR1 was essentially excluded from the lipid raft fraction.

Interaction of GLUT1 with stomatin. Our initial attempt to demonstrate an interaction between GLUT1 and stomatin in normal 3T3-L1 adipocytes in coprecipitation experiments was not successful. This observation is not unusual on the basis of the fact that the interacting pools are likely small and most investigators examine the interaction between proteins when one or both of the partners are overexpressed. We thus pursued overexpression of stomatin using the Moloney murine leukemia virus expression vector. Figure 4 shows that overexpression was successful on the basis of Northern (Fig. 4A) and
Probed only for stomatin expression.

Western blot analysis (Fig. 4B). In Fig. 4A, endogenously expressed stomatin mRNA (2,821 bases) was observed in control, GFP-overexpressing, and stomatin-overexpressing cells. In addition to this form, stomatin-overexpressing cells contained two additional bands: one that was higher than the endogenous message and one that was lower. The transcription start site in the retroviral vector adds ~1,500 bp 5’ to the stomatin coding sequence (~1,000 bp). The polyadenylation site of the vector is ~600 bp 3’ to the stomatin coding sequence. Thus we would expect an mRNA band at ~3,200 bases, which is represented in lane 3 by the upper band. pBMN has splice donor and acceptor sites in the packaging domain that are ~1,000 bp apart. If this splice occurs, then we would expect an mRNA band at ~2,200 bases, which is observed in lane 3. Despite the difference in the sizes of the message, the migration of overexpressed stomatin protein was identical to that of endogenously expressed stomatin (Fig. 4B).

We also examined the subcellular distribution of stomatin and GLUT1 in overexpressing cells. Figure 4C shows that stomatin distribution in overexpressing cells is similar to that in control cells, although a small proportion of the pool is located in the LDM fraction. In neither the HDM nor the PM fractions did fructose affect expression. Likewise, the distribution of GLUT1 was altered by stomatin overexpression (data not shown). With these cells, we next examined the colocalization of GLUT1 and stomatin in isolated PM vesicles (Fig. 5). In these experiments, we exposed PM vesicles to anti-stomatin antibodies attached to magnetic beads and then collected the stomatin-containing vesicles with a magnetic concentrator. We (13) have shown previously that these vesicles are distributed about equally between inside-out and outside-out orientations. Thus we expected that 50% of the stomatin-containing vesicles would be collected by the antibody, given that the epitope for the stomatin antibodies is within the cytoplasmic domain. This was confirmed by examining the adsorbed vs. the nonadsorbed membranes (compare Fig. 5B with Fig. 5C). Importantly, this shows that the antibody collects nearly 100% of the stomatin-containing vesicles in the appropriate orientation. These vesicles also contained GLUT1 on the basis of Western blotting analysis (Fig. 5A). Interestingly, about twice as much GLUT1 was associated with these vesicles when derived from PM isolated from fructose-fed compared with glucose-fed cells.

Our next goal was to immunoprecipitate GLUT1 from detergent-extracted cells. Because our protocol calls for C12E9 extraction, we first determined whether this detergent disrupts lipid rafts and fully solubilizes GLUT1 and stomatin. These data are shown in Fig. 6. Figure 6A represents lysates from normal 3T3-L1 adipocytes applied to a sucrose gradient, whereas Fig. 6B represents lysates from stomatin-overexpressing 3T3-L1 adipocytes. With TX-100 extraction of both normal and stomatin-overexpressing cells, the detergent-resistant membranes (lipid rafts) were collected in fraction 3. A portion of both the GLUT1 and stomatin pools was associated with this fraction. Although more stomatin was present in the overexpressing cells, as expected, overexpression per se did not influence the relative distribution of either GLUT1 or stomatin between the detergent-resistant and detergent-soluble membranes. In C12E9-extracted cells, both GLUT1 and stomatin shifted to higher-density fractions compared with extraction...
with TX-100. Thus C$_{12}$E$_{9}$ efficiently solubilizes GLUT1 and stomatin such that coprecipitated material will represent specific interactions rather than association by inefficient extraction (i.e., contained within the detergent-resistant membranes). Indeed, in stomatin-overexpressing cells, but not in GFP-overexpressing cells, stomatin was coprecipitated with GLUT1 (Fig. 7A). Fructose feeding increased the amount of stomatin that coprecipitated with GLUT1 approximately threefold (the relative density of stomatin in glucose-fed cells was 0.14 ± 0.05 compared with 0.44 ± 0.104 in fructose-fed cells; P = 0.03, n = 5). This interaction was stable to high-salt washes. The blot was reprobed for GLUT1 to demonstrate that GLUT1 was indeed immunoprecipitated (Fig. 7B). The strong intensity of the bands, despite limited exposure to film (<30 s), represents the fact that >85% of the GLUT1 pool from 10$^{10}$ cells is collected during immunoprecipitation with a single round of antibody exposure (32). To detect the pool of stomatin which did not coprecipitate with GLUT1, the leftover extracts were exposed to anti-stomatin antibodies. The immune complexes were again collected and processed by Western blotting (Fig. 7C). The residual stomatin was detected as very dark bands, which suggests that only a small proportion of the stomatin pool actually interacts with GLUT1.

**Effect of stomatin overexpression on glucose transport activity.** Finally, we determined the effect of stomatin overexpression on transport activity. Because the differentiation is not as complete in the virally infected cells as in normal cells (30), we have reported the activity data on the basis of protein concentration. As shown in Fig. 8, transport activity was significantly elevated in all cells when chronically treated with fructose compared with glucose. The difference in transport activity between the fructose-treated control cells and those virally infected was statistically significant. However, there was no statistical difference between fructose-treated, stomatin-overexpressing cells and GFP-overexpressing cells, the latter of which we believe is a more suitable control. With regard to basal glucose uptake (i.e., in the presence of glucose), there was no statistical difference between the control and the virally infected cells. The transport rate between the GFP-overexpressing cells (0.66 ± 0.04 nmol·mg protein$^{-1}$·min$^{-1}$) and stomatin-overexpressing cells (0.51 ± 0.05 nmol·mg protein$^{-1}$·min$^{-1}$) was indeed significantly elevated in cells chronically treated with fructose compared with glucose.

**Fig. 7.** Stomatin coprecipitates with GLUT1 from whole cell lysates. GFP-overexpressing (GFP), and stomatin-overexpressing (Stomatin) 3T3-L1 adipocytes were incubated in 25 mM glucose or 25 mM fructose for 24 h. Cells from individual 10-cm dishes were extracted in 900 µl of lysis buffer [2% C$_{12}$E$_{9}$, 0.1% SDS, 1 mM PMSF, and protease inhibitor cocktail (Sigma)] and placed on ice for 30 min. Lysates were clarified by centrifugation at 3,000 g for 5 min. Resulting supernatants were preincubated with preimmune serum for 1 h and then removed with protein A-Sepharose. Supernatant was then exposed to peptide-puriﬁed anti-GLUT1 IgG (10 µg) for 4 h at 4°C. Immune complex was collected with protein A-Sepharose at 4°C overnight, washed with lysis buffer (in absence or presence of 1 M NaCl), and released with Laemmli sample dilution buffer in the presence of mercaptoethanol at 37°C for 30 min. Extract was further processed by incubating with anti-stomatin antibodies overnight. Immune complexes were collected with protein A-Sepharose, washed with buffer (in the absence of NaCl), and released with Laemmli sample dilution buffer in the presence of reducing agent. Proteins were separated on a 12.5% SDS-PAGE gel and transferred to nitrocellulose. A: blot was processed with anti-stomatin and the interaction detected using ECL reagents. Note higher molecular mass immunoreactive species in A. These represent heavy- and light-chain IgG, which are detected by the secondary antibody. B: the same blot was then reprocessed, without stripping, with anti-GLUT1 antibodies. HS, immunoprecipitates washed with 1 M NaCl. C: Western blotting for stomatin, immunoprecipitated from extracts after GLUT1 precipitation. Stomatin is indicated by arrow. Migration of GLUT1 is indicated by the bracket. Molecular mass markers are shown in kDa. Reduced IgG is indicated by the arrows on left.

**Fig. 8.** Effect of stomatin overexpression of glucose transport activity. Normal 3T3-L1 adipocytes and those overexpressing GFP or stomatin were exposed to medium containing 25 mM glucose (filled bars) or 25 mM fructose (hatched bars) for 24 h. Transport activity was measured over a 10-min interval, as described in MATERIALS AND METHODS. Data represent the average ± SE of 4 (control) or 6 (GFP- and stomatin-overexpressing cells) independent experiments, each done in duplicate. Cytochalasin B-inhibitable transport was 95% of total activity. **P < 0.01 vs. fructose-fed control cells; *P = 0.05 vs. glucose-fed, GFP-overexpressing cells.
protein \(^{-1}\)min\(^{-1}\) was statistically different but borderline \((P = 0.05)\). We conclude from these data that stomatin overexpression has little effect on either basal or activated glucose uptake.

**DISCUSSION**

In this study, we have investigated the possible mechanisms that underlie the increase in glucose transport activity in 3T3-L1 adipocytes in response to glucose deprivation. We have developed a glucose deprivation protocol that depletes the cells of intracellular glucose while preventing aberrant protein glycosylation, including that of GLUT1 (27). In the current experiments, we confirmed that neither the total pool of GLUT1 nor that which resides in the PM changes in response to glucose deprivation (i.e., cells supplemented with fructose). However, we now show that glucose deprivation does alter the distribution of GLUT1 within the PM. Specifically, fructose feeding causes a reversible translocation to lipid rafts, detergent-resistant domains within the PM. Although the functional consequence of this reorganization has not been ascertained, it is correlated with the increase in glucose uptake associated with glucose deprivation. This represents the first time that GLUT1 redistribution within the PM has been reported in response to the availability of its own substrate. We also show that GLUT1 interacts with stomatin, a type II membrane protein, in 3T3-L1 adipocytes, which confirms the interaction observed in Clone 9 cells by Zhang et al. (62). However, ours is the first study to demonstrate a change in that association in response to conditions that alter transport activity. Specifically, glucose deprivation increases the association between GLUT1 and stomatin as determined by coprecipitation assays. We favor the concept that this interaction occurs in lipid rafts, because GLUT1 and stomatin colocalize to this compartment and glucose deprivation increases the pool of GLUT1 in this subdomain of the PM.

The concept of lipid domains as organizing centers in the PM has gained acceptance in recent years. Championed by Simons and Ikonen (48), there is strong evidence that these domains, called lipid rafts, sequester signaling molecules, such as GPI-anchored proteins, transmembrane proteins, and diacylated proteins. These rafts are enriched in sphingolipids and cholesterol and are found in two distinct locations. One type of lipid raft is found in “free-floating” subdomains in the PM; the other is contained in caveolae. Caveolae, a term coined by Anderson et al. (1), are invaginations of the PM that are particularly abundant in lung and adipose (11). These structures are coated with a 22-kDa membrane protein called caveolin, originally identified by Glenney (16). Proteins contained within both the free-floating and caveolae-containing lipid rafts are resistant to TX-100 extraction at 4°C. The glycolipid-enriched complexes float in sucrose density gradients, which affords a method of separation and identification from peripheral membrane protein and detergent-soluble proteins, the technique used in our study to identify lipid rafts.

A role for caveolae/lipid rafts in glucose transport has been debated over the past decade. The possible participation of caveolae in GLUT4 trafficking was initially proposed in the early 1990s on the basis of observation that caveolae-enriched membrane fractions from 3T3-L1 adipocytes contained GLUT4 (45). However, no colocalization could be demonstrated between GLUT4-containing vesicles and caveolin in isolated rat adipocytes (21) or skeletal muscle (34), which questioned the observation in 3T3-L1 adipocytes. Recently, however, the caveolin issue has been revisited by several groups of investigators (20, 22, 40). Saltiel’s group [Baumann et al. (3)] have shown that activated insulin receptor kinase phosphorylates the protooncogene, Cbl. The phosphorylated Cbl is recruited to caveolae through an adaptor [Cbl-associated protein (CAP)], a step required for insulin-stimulated glucose transport. The same group has shown that CAP is required for the recruitment of TC10, a Rho family GTPase, to lipid rafts [Chiang et al. (7)] and that this compartmentalization is also required for GLUT4 translocation to the PM [Watson et al. (58)]. Finally, they have demonstrated that the sorbin homology domain in CAP is required for binding to the caveolae resident protein flotillin [Kimura et al. (23)]. Flotillin is a member of the Band 7.2/stomatin protein family (10). Identified by its association with TX-100-insoluble mouse lung proteins (4), it has only 18% sequence similarity to stomatin but has a similar structure.

Our data show for the first time that stomatin is expressed in an adipocyte model system, confirming the ubiquitous expression of this protein. Glucose deprivation has little effect on stomatin expression or compartmentation in either control or stomatin-overexpressing cells. As expected from recent studies (35, 43), we found that a portion of the stomatin pool resides in lipid rafts. In isolated PM, this represents 90% of the pool. Although overexpression of stomatin did not influence transport activity, the enhanced interaction with GLUT1 suggests that stomatin may play a role similar to that of flotillin in the recruitment of proteins to lipid rafts.

Two other reports have recently shown that GLUT1 is associated with lipid rafts. Sakyo and Kitagawa (42) have shown that GLUT1, but not GLUT3, is associated, in part, with lipid rafts in both tumorigenic and nontumorigenic human cell hybrids. Although the functional consequence of this segregation was not investigated, those authors suggested that specific sorting signals may define the localization of these proteins. Furthermore, this may indicate separate roles for these transporters. Rubin and Ismail-Beigi (41) have also shown the localization of GLUT1 to lipid rafts in Clone 9 cells, a localization that is reduced after azide treatment. Because azide acutely activates glucose transport in these cells (47), these authors suggest that it is the loss of GLUT1 from this compartment that underlies this regulation. Although this group has shown that stomatin overexpression decreases GLUT1 activity (61), they did not determine whether azide interferes with this interaction. Regardless, our results are essentially opposite to these. We must point out, however, that the effects of azide are tested under acute conditions in an unrelated model system, which may underlie the differences observed in 3T3-L1 adipocytes. Our data support the hypothesis that glucose deprivation stimulates the redistribution of GLUT1 to stomatin-containing lipid rafts, an environment that favors activation.

To investigate the mechanism(s) that underlie GLUT1 translocation to lipid rafts and possible transformation from the inactive to the active state, we are considering the following possibilities, which include palmitoylation, phosphorylation, and/or oligomerization. Many of the proteins associated with lipid rafts are, or become, palmitoylated. Caveolin is palmitoylated on multiple cysteines, although this modification is not
necessary for localization to caveolae (9). Stomatin is also palmitoylated (51), although the requirement of palmitoylation for its association with lipid rafts seems unlikely on the basis of its structural similarity to caveolin. In contrast is the palmitoylation of Src family members, in which Cys3 palmitoylation is both necessary and sufficient to confer localization to caveolae (46). GLUT1 has six cysteine residues, several of which are predicted to have access to the cytoplasmic compartment (33). This localization favors palmitoylation (60). Indeed, Poulriot and Beliveau (38) have demonstrated that GLUT1 in brain capillaries is palmitoylated. Interestingly, palmitoylation of GLUT1 was increased in both diet-induced hyperglycemic and diabetic animals. The relevance of palmitoylation to transport activity was not assessed in this study although, in general, hyperglycemia reduces glucose uptake.

Proteins targeted to lipid rafts can also be phosphorylated. Caveolin itself is phosphorylated on tyrosine, which is correlated with transformation of fibroblasts by Rous sarcoma virus (16). Recently, Kimura et al. (24) have shown that the insulin receptor associates with and catalyzes the tyrosine phosphorylation of caveolin. Although the consequence of tyrosine phosphorylation could not be studied, those authors speculated that the phosphorylated caveolin recruits signaling molecules to caveolae. Furthermore, Sowa et al. (52) demonstrated that the phosphorylation of caveolin 2 modules caveolin 1-dependent caveola formation. Inhibitors of casein kinase 2 blocked phosphorylation, implying the involvement of this kinase. Stomatin can also be phosphorylated, but in response to cAMP (57). Once again, the functional significance of this modification is unknown. With regard to GLUT1, we have been unsuccessful in our attempts to detect phosphorylated GLUT1 in glucose-deprived cells, but we have no positive control for in vivo GLUT1 phosphorylation.

Finally, it is possible that oligomerization is responsible for targeting GLUT1 to lipid rafts. Certainly, caveolin (44) and stomatin (50) form oligomeric structures, which may be important for their organization within the PM. Carruthers and colleagues [Hebert and Carruthers (18) and Pessino et al. (36)] have hypothesized that GLUT1 also forms oligomers. Recent publications from this group suggest that the active form of the transporter in red blood cells is a homotetramer that is sensitive to both ATP and reductant (see Ref. 8 for overview). They propose that the homotetramer simultaneously presents two glucose entry sites and two exit sites. ATP binding, to the putative cytoplasmic loop 5, reduces both the $k_{\text{max}}$ and $K_m$ 10- to 20-fold, respectively, with an increase in cytochalasin B binding. This suggests that ATP binding favors the exposure of the cytoplasmic glucose-binding sites in the homotetramer, which essentially blocks glucose uptake. Reducing equivalents also block glucose uptake but do so by dissociating the homotetramer into two dimers. From this, we might speculate that the glucose-fed 3T3-L1 adipocytes contain predominantly GLUT1 dimers and that glucose deprivation causes association of these dimers, which creates a more active complex. As the tertiary structure of GLUT1 is sensitive to its environment, perhaps the composition of lipid rafts favors this association. Alternatively, GLUT1 could exist in the homotramer configuration but bound to ATP in glucose-fed cells. Glucose deprivation might induce the dissociation of ATP from GLUT1, which could enhance glucose uptake. Although ATP concentration does not appear to change in response to glucose deprivation in 3T3-L1 adipocytes (56), there could be local changes in ATP concentration that cause its dissociation from GLUT1. Again, lipid rafts offer a compartment that might promote such a change.

ACKNOWLEDGMENTS

We thank Xiao Wei Gu for excellent assistance in cell culture.

GRANTS

This work was funded in part by the National Institute of Diabetes and Digestive and Kidney Diseases (DK-45035) and the American Heart Association.

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