Translocation of GLUT1 Does Not Account for Elevated Glucose Transport in Glucose-deprived 3T3-L1 Adipocytes*

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Glucose deprivation increases the rate of glucose transport in 3T3-L1 adipocytes in a protein synthesis-dependent fashion. To determine if translocation of either GLUT1 or GLUT4 is responsible for this phenomenon, we adapted existing fractionation procedures toward isolating 3T3-L1 adipocyte membranes. By Western blot analysis of equal protein, GLUT1 was distributed between plasma membranes, high density “microsomal” membranes, and low density “microsomal” membranes isolated from control cells. GLUT4 comigrated with high density and low density membranes. Glucose deprivation for 12 h did not alter the distribution of either GLUT1 or GLUT4, despite an 8-10-fold increase in glucose transport activity in intact cells. Importantly, increased transport activity was retained in plasma membrane vesicles isolated from glucose-deprived cells. These data show for the first time that the increase in transport activity associated with glucose deprivation does not result from the translocation of either of the glucose transporters known to exist in 3T3-L1 adipocytes. As GLUT4 is excluded from the plasma membrane, these data provide evidence for activation of GLUT1.

The 3T3-L1 cell line has provided an important tool in the study of glucose transport because this adipocyte model system allows the investigation of chronic treatments. This has been a distinct advantage in examining the effects of glucose deprivation which increases the rate of glucose transport in a time- and protein synthesis-dependent manner (1-3). Although the deprivation phenomenon has been documented in numerous cell types, it is evident that cell type-specific mechanism(s) define the increase in transport activity, as reviewed recently (4). In chick embryo fibroblasts (5), glia (6), myocytes (7), and L6 muscle cells (8), an increase in GLUT1 mRNA results in greater levels of GLUT1 protein for insertion into the plasma membrane, although this latter aspect has not been tested. In murine fibroblasts (9, 10), GLUT1 increases not as a function of increased message availability but via a block in GLUT1 degradation. A new equilibrium presumably is established between GLUT1 stored inside the cell and that expressed on the cell surface, although again, this has not been measured. In our hands, the amount of GLUT1 in 3T3-L1 adipocytes does not change during the time in which transport activity increases (11), confirming the earlier work of Reed et al. (2). With extended deprivation, the level of GLUT1 does increase but as a function of the accumulation of an aberrant form of GLUT1 (11). Importantly, the appearance of the lower molecular weight glycoform of GLUT1 does not correlate with further changes in transport activity. A second consideration in 3T3-L1 adipocytes is the contribution to transport activity by GLUT4, the insulin-responsive glucose transporter. While the levels of GLUT4 mRNA fall during glucose deprivation (3, 11), it has been reported that GLUT4 protein expression is unchanged (3). From these observations, two mechanisms might underlie the deprivation-induced increase in transport activity: translocation of either GLUT1 or GLUT4 from intracellular storage compartments or activation of pre-existing cell surface transporters. Our goal in the present study was to examine translocation, specifically.

Many investigators using 3T3-L1 adipocytes employ some modification of a technique developed by Simpson et al. (12) for the subfractionation of isolated rat adipocytes. However, the many variations in the literature indicate the difficulty inherent in the reliable subfractionation of these cells. To determine the role of translocation in the glucose deprivation-induced elevation in transport activity, we first required a dependable method for 3T3-L1 subfractionation. We accomplished this using a steel block, ball-bearing homogenizer designed by Balch and Rothman (13) for the purification of endosomes, in combination with a detailed subfractionation procedure reported by Weber et al. (14) for isolated rat adipocytes. With this combination, we demonstrate that about 20% of GLUT1 resides at the cell surface in control cells and that its distribution does not change in response to glucose deprivation. Furthermore, only about 3% of GLUT4 resides at the cell surface in basal cells which, like GLUT1, does not change upon glucose deprivation. We conclude that recruitment of transporters, either GLUT1 or GLUT4, is not responsible for the increase in transport activity in response to glucose deprivation.

EXPERIMENTAL PROCEDURES

Materials—Dulbecco’s modified Eagle’s medium and glucose-free Dulbecco’s modified Eagle’s medium were obtained from Life Technologies, Inc. (Gaithersburg, MD). Calf serum (J13605) and fetal bovine serum (LB95508) were purchased from Intergen (Milford, MA). Rabbit polyclonal antibodies for GLUT1 and GLUT4 were generated against the C-terminal 13 amino acids, respectively. The anti-GRP78 antibody was generated against a peptide containing the first 12 amino acids in the N terminus. Anti-sialyltransferase was a gift from Dr. William Dunn (University of Florida). Anti-Na⁺,K⁺-ATPase was a gift from Dr. Michael Caplan (Yale University). NHS-LC-biotin was purchased from Pierce (Rockford, IL). The steel block, ball bearing homogenizer was made by Auburn Tool and Die (Warwick, RI). All other reagents used were of analytical grade from commercial sources.

Cell Culture, Glucose Deprivation, Whole Cell Glucose Transport, and Western Analysis—Techniques for these methodologies have been de-
RESULTS AND DISCUSSION

We (11) and others (1–3) have shown previously that glucose deprivation increases the rate of glucose transport in 3T3-L1 adipocytes. Although we have used total glucose deprivation to maximize the activation phenomenon, this observation is of physiological relevance because fluctuation of transport activity underlies the increase in transport activity within the biological limits of circulating glucose (13). As reported by Weber (14), a combination of differential and sucrose gradient centrifugation provided the means by which the three subcellular membrane fractions were separated. Final pellets were resuspended in 0.3 ml of TES. Protein recovery from subfractionation and subcellular fractions from the control and glucose-deprived cells was similar (Table I). Including the mitochondrial/nuclear fraction, membrane constituted 35% of the recovered protein.

To determine the efficiency of the fractionation procedure, we utilized two approaches. First, we followed the distribution of plasma membranes by biotinylating cell surface proteins prior to subfractionation and second, we examined the appearance of selected proteins chosen for their presence in PM or intracellular membranes. Biotinylated proteins were enriched in the PM fraction over the HDM and LDM (Fig. 2). To quantify this enrichment, we used a dot-blot analysis of equal protein (50 ng) from each fraction. Relative to homogenate, the PM fraction was enriched in biotinylated proteins by 12-fold. This enrichment was similar to the increase in specific activity of 5'-nucleotidase, a marker enzyme for plasma membranes, noted by Weber and colleagues (14). Based on the total recovery of protein in each fraction, less than 2% of biotinylated proteins comigrated with the LDM fraction, while approximately 10% of the recovered protein was coeluted with 35% in the PM fraction (Table I).

Effect of glucose-deprivation on uptake of [3H]2-deoxyglucose. 3T3-L1 adipocytes were glucose-fed or glucose-deprived (in the presence of fructose) for 12 h before subfractionation. The total amount of protein for homogenates and membrane fractions was determined from four independent subfractionation experiments and is reported as S.E.

| Fraction      | Fed | Glucose-deprived |
|---------------|-----|------------------|
| Homogenate    | 33.2 ± 2.9 | 31.0 ± 3.0 |
| PM            | 0.42 ± 0.05 | 0.41 ± 0.06 |
| HDM           | 0.66 ± 0.08 | 0.64 ± 0.09 |
| LDM           | 1.22 ± 0.25 | 1.34 ± 0.14 |

Fig. 1. Effect of glucose-deprivation on uptake of [3H]2-deoxyglucose. 3T3-L1 adipocytes in 35-mm plates were incubated for 12 h in medium in the absence (S) or presence (F) of 25 mM glucose, or with 25 mM fructose in a glucose-free environment (+ Fru). Transport of deoxyglucose was assayed for 10 min as described previously (11). The rates, reported as nmol/10⁵ cells/min ± S.E., are for duplicate assays and are representative of two independent experiments.

Fig. 2. Distribution of cell-surface biotinylated proteins. Cells were incubated for 12 h in medium in the presence of 25 mM fructose in a glucose-free background (S) or 25 mM glucose (F). After biotinylation, cells were subfractionated as described in the text. Each lane represents 25 μg of protein. Samples of homogonate (H) from non-biotinylated (−) and biotinylated (+) cells were loaded as controls for comparison to HDM, LDM, and PM. Biotinylated proteins were detected with streptavidin–horseradish peroxidase using enhanced chemiluminescence. This figure is representative of two independent experiments.
biotinylated proteins comigrated with the HDM fraction. We confirmed that 10% of the PM proteins migrated with the HDM fraction by analysis of Western blots using an antibody against the α-subunit of Na⁺,K⁺-ATPase, a plasma membrane resident protein, which gave identical results (Fig. 3). To analyze contamination of the PM fraction with membranes of intracellular origin, we measured the relative amount of GRP78, an endoplasmic reticulum chaperone, sialyltransferase, a trans-Golgi marker, and GLUT4, an endosomal marker (16), in membrane fractions using antibodies specific for each protein. GRP78 was localized predominantly to the HDM fraction, with about 12% cross-over to the PM (Fig. 3). These data are identical to those reported for cytochrome c reductase by Weber et al. (14). Sialyltransferase was also localized to the HDM fraction with less than 2% comigrating with the PM fraction (data not shown). Only 3% of GLUT4 colocalized with the PM fraction in the presence or absence of glucose (Fig. 3, Table I), demonstrating that the PM is relatively free from endosomal contamination. This suggests that GLUT4 contributes little to basal transport and that it does not underlie the mechanism of transport activation.

We then examined the distribution of GLUT1 in membrane fractions isolated from glucose-fed and glucose-deprived cells (Fig. 3A). Densitometric analysis of GLUT1, including the mitochondrial/nuclear fraction, revealed 70% recovery of GLUT1 relative to the homogenate. Fig. 3 shows GLUT1 distribution among the HDM, LDM, and PM fractions only, as these fractions contain 12–19 times more GLUT1 than the mitochondrial/nuclear fraction. Based on protein recovered in each of these fractions, we calculated that 20% of GLUT1 resides on the cell surface (Table II), similar to that measured by cell-surface photolabeling (17). With glucose deprivation, even if the entire pool of GLUT1 translocated to the PM, we would predict only a 5-fold change in transport activity. Importantly, glucose deprivation did not alter the localization of GLUT1 (Fig. 3A). As a positive control for translocation, we have included an experiment in which control cells were acutely stimulated with insulin (Fig. 3B). With a 10-min exposure to 1 μM insulin, the decrease in GLUT1 in the intracellular fractions (40%) corresponded to a nearly equal increase in the PM. GLUT4 content was reduced in the LDM by about 50%. The densitometric value for the loss in the LDM fraction was nearly equal to that which translocated to the PM. These observations are similar to previously published data on the insulin-induced translocation of the glucose transporters in 3T3-L1 adipocytes (18, 19). Therefore the subfractionation procedure described here provides a valid means for the detection of translocation events.

It was of interest to determine whether PM vesicles isolated from glucose-deprived cells retain elevated transport activity. In Fig. 4, we compare vesicle transport data derived from glucose-deprived versus glucose-fed PM, using cytochalasin B to correct for nonspecific transport. No difference in transport activity between glucose-fed and glucose-deprived vesicles was seen in the presence of cytochalasin B, suggesting that vesicle volume does not change. The results indicate that transport in glucose-fed PM vesicles was below the limit of detection. However, we consistently observed transport in glucose-deprived PM vesicles as a significant increase over background.

In conclusion, we have shown here that GLUT1 is the major transporter in the PM in both glucose-fed and glucose-deprived 3T3-L1 cells. This establishes that translocation of GLUT1 from intracellular membranes to the PM is not responsible for elevated transport activity in response to glucose deprivation. Furthermore, we have demonstrated that PM vesicles isolated from glucose-deprived cells exhibit elevated transport activity. Based on the protein synthesis-dependent nature of the increase in transport activity, we hypothesize the involvement of a novel protein in transport activation. This protein could function either by activating GLUT1 or by interrupting an association between the transporter and an inhibitor molecule. In future experiments, we shall utilize the subfractionation procedure featured here to identify plasma membrane proteins unique to glucose-deprived cells as potential candidates for transport binding proteins.

Acknowledgment—We thank Mike Thomson for providing one set of the +/- insulin transporter distribution results.

**Table II**

| Transporter | Condition | HDM | LDM | PM |
|-------------|-----------|-----|-----|----|
| GLUT1       | C         | 38.9 ± 2.6 | 40.7 ± 4.5 | 20.4 ± 2.3 |
|             | S         | 37.0 ± 0.2 | 45.1 ± 0.3 | 17.9 ± 0.1 |
| GLUT4       | I         | 42.4 ± 1.4 | 26.0 ± 2.8 | 31.6 ± 2.7 |
|             | C         | 31.8 ± 3.3 | 65.5 ± 3.8 | 26.0 ± 0.7 |
|             | S         | 29.2 ± 0.7 | 67.9 ± 3.2 | 29.2 ± 2.5 |
|             | I         | 45.8 ± 3.8 | 32.2 ± 5.6 | 22.0 ± 5.6 |

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