Glut4 Is Targeted to Specific Vesicles in Adipocytes of Transgenic Mice Overexpressing Glut4 Selectively in Adipose Tissue*

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Adipocytes of transgenic mice overexpressing Glut4 selectively in adipose tissue (Shepherd, P. R., Gnudi, L., Tozzo, E., Yang, H., Leach, F., and Kahn, B. B. (1993) J. Biol. Chem. 268, 22243–22246) have 15–20-fold more Glut4 than normal adipocytes. To study compartmentalization of intracellular Glut4 in these cells, we fractionated light microsomes prepared from transgenic and normal adipocytes in velocity and density sucrose gradients. Glut4-containing intracellular membranes from both cell types have a specific and narrow distribution in these gradients, i.e. behave as homogeneous vesicles with identical sedimentation coefficients and different buoyant densities. Immunoadsorption of Glut4-containing vesicles with covalently immobilized monoclonal anti-transporter antibody demonstrated that the total polypeptide composition of these vesicles from transgenic and normal cells was identical, with the exception of Glut4 itself, which was much more abundant in the transgenic cells. Both preparations also had comparable levels of secretory carrier membrane proteins and of aminopeptidase activity (gp160). Glut4-containing vesicles from both normal and transgenic adipocytes excluded Glut1, which in both cell types formed a different vesicle population. Thus, even under conditions of high level overexpression, Glut4 is still specifically targeted to the same unique type of structurally defined insulin-sensitive vesicles as in normal cells.

Insulin-sensitive adipose and skeletal muscle tissues express a specific isoform of glucose transporter protein, Glut4, which under basal conditions, i.e. in the absence of insulin, resides in intracellular microsomal vesicles. After insulin administration, Glut4-containing vesicles fuse with the plasma membrane and deliver the transporter to the cell surface (for recent reviews, see Birnbaum (1992), Bell et al. (1993), Mueckler (1994), James and Piper (1994), James et al. (1994), Holman and Cushman (1994), and Stephens and Pilch (1995)). It is likely that this process largely accounts for insulin's effect on blood glucose update. Therefore, transporter translocation has enormous physiological significance, and its molecular mechanism has been studied in many laboratories. Important questions in this regard are how Glut4 is retained inside a cell and how its movement to the cell surface is regulated by insulin, while other members of the glucose transporter family stay permanently at the plasma membrane and are not insulin-regulatable. A general agreement has been reached that the C terminus of Glut4 defines its intracellular localization (Czech et al., 1993; Verhey et al., 1993; Marshall et al., 1993; Haney et al., 1995), although this is not a unanimous conclusion (Piper et al., 1992, 1993; Asano et al., 1992). The nature of the Glut4-containing intracellular compartment(s) and the mechanism of its insulin-sensitive translocation to the cell surface remain unknown.

To define the nature of this compartment, it will be important to understand its relation to various other intracellular membrane structures and, in particular, to transport vesicles that shuttle between different subcellular membrane structures such as the endoplasmic reticulum, Golgi apparatus, and plasma membrane. We need to determine whether Glut4-containing structures represent unique and specialized subcellular particles (organelles?) or a subpopulation of Golgi-to-plasma membrane transport vesicles, a component of the normal endocytotic pathway, or something else. Immunocytochemical studies of Glut4 in fat and muscle cells (Smith et al., 1991; Slot et al., 1991a, 1991b; Rodnick et al., 1992) revealed some of the morphological features characteristic of Glut4-containing structures, but still left this question open. Biochemical studies demonstrated that Glut4-containing vesicles from adipose and muscle tissues are very homogeneous in size, sedimentation coefficient, buoyant density, and total polypeptide composition (Kandror et al., 1995a); have at least one marker protein besides Glut4, namely the aminopeptidase gp160 (Kandror and Pilch, 1994b; Kandror et al., 1994); and thus may represent a unique specialized compartment.

The development of transgenic mice overexpressing Glut4 selectively in fat tissue (Shepherd et al., 1993) provided an excellent physiological model for studies on compartmentalization and targeting of the transporter. Transgenic mice give the advantages of transfection experiments and still allow for studies with native adipocytes instead of less differentiated or undifferentiated cell lines.

The central question addressed in this study was whether overexpressed Glut4 was compartmentalized in the specific vesicles, as in normal adipocytes, or whether it “spilled” out into other cellular membranes. The data that we present here are consistent with the interpretation that overexpressed Glut4 is retained in a specific vesicle population that is biochemically homogeneous to intracellular Glut4 vesicles from nontransgenic adipocytes.
**Figure 1.** Coomassie Blue staining of (A) and Glut4 content in (B) light microsomes from transgenic and nontransgenic adipocytes. 15 μg of light microsomes from transgenic (tg) and nontransgenic (ntg) adipocytes were electrophoresed in a 7.5% polyacrylamide gel. A, Coomassie Blue staining of the gel, with molecular masses of the protein standards shown on the right; B, Western blotting of the same preparation with anti-Glut4 monoclonal antibody 1F8 after transfer to polyvinylidene difluoride membrane. Results are representative of five different experiments with 10–15 mice per experiment.

**MATERIALS AND METHODS**

Antibodies—In this study, we used the monoclonal anti-Glut4 antibody 1F8 (James et al., 1988), polyclonal anti-Glut1 antibody (a gift from Dr. Bernard Thorens), monoclonal anti-GTV3/SCAMP1 antibody (Thöidis et al., 1993), and monoclonal anti-caveolin antibody (Transduction Laboratories).

Transgenic Animals—Transgenic mice were engineered by injecting the following DNA construct into the pronucleus of fertilized zygotes from FVB mice and transfer to pseudopregnant females. The adipose-specific Glut4 transgene was constructed as described previously using the 5.4-kilobase adipose-specific promoter/enhancer (a gift of Drs. B. M. Spiegelman and R. Graves) from the fatty acid-binding protein gene ap2 ligated to a 6.3-kilobase BamHI-PvuI genomic DNA fragment corresponding to bases 2063–8396 of the human GLUT4 gene (a gift of Drs. J. Buse and G. I. Bell). The sequence contains all exons and introns and a consensus polyadenylation signal of Glut4. To identify transgenic mice, DNA was extracted from tail clippings and subjected to Southern blotting or polymerase chain reaction using primers 5'-TAT-CAT-CTC-TCA-CTG-GCT-TGG-AAG-3' and 5'-CTG-GCT-GGT-CGA-ATA-ATA-G-3'. Mice were housed at 21°C with a 12-h light/dark cycle and were fed standard Purina mouse chow 5008 ad libitum. All studies were carried out in heterozygous mice at 12–14 weeks of age. Results were confirmed in both male and female mice.

Preparation of Light Microsomes from Adipose Cells—Subcellular fractionation of adipocytes was performed as described by Simpson et al. (1983). Briefly, adipocytes were isolated from gonadal fat pads of 10–15 mice per genotype for each experiment by collagenase digestion (1 mg/ml). Fat pads were incubated at 37°C with constant shaking in Krebs-Ringer phosphate buffer (2 ml/g of fat) with 20 mM Hepes, 2.5% (1 mg/ml). Fat pads were incubated at 37°C with constant shaking in Krebs-Ringer phosphate buffer (2 ml/g of fat) with 20 mM Hepes, 2.5% bovine serum albumin (fraction V), and 200 mM adenosine (pH 7.4). Cells were washed four times with buffer and twice with TES to reduce the bovine serum albumin content and homogenized, and light microsomes (LM) were prepared.

Isolation of Glut4-containing Vesicles—This was carried out by immunosorption of light microsomes on monoclonal anti-Glut4 antibody 1F8 immobilized on polyacrylamide beads. Nonspecific adsorption of microsomes was monitored by passage of membranes over IgG beads and with 5% bovine serum albumin content and homogenized, and light microsomes (LM) were prepared.

**RESULTS**

The fraction of intracellular LM that is enriched in Golgi apparatus protein markers (Simpson et al., 1983) was isolated from nontransgenic and transgenic adipocytes as described under "Materials and Methods." Fig. 1 (A and B) demonstrates by Coomassie Blue staining and Western blotting that transgenic mice contain at least 15–20 times more Glut4 in this fraction in comparison with normal control mice, whereas the total...
polypeptide composition of their LM is not changed. This demonstrates that overexpression of Glut4 is highly specific and does not cause major perturbations in the protein composition of microsomal membranes. Our further work was directed toward comparative characterization of Glut4-containing compartments from adipocytes of transgenic and normal mice.

We have previously demonstrated that Glut4-containing vesicles from rat adipose and muscle tissues behave as individual particles with a sedimentation coefficient of $-100$–$120$ S (Kandror and Pilch, 1994b; Kandror et al., 1995a). So, in the next experiment (Fig. 2, A and B), we compared the sedimentation behavior of Glut4-containing structures from normal and transgenic mice. As expected, the total protein distribution was similar in both gradients (Fig. 2A), whereas the amount of Glut4 was much higher in the vesicles from transgenic mice (Fig. 2, B and C). The striking result of this experiment was that the sedimentation coefficients of Glut4-containing structures from transgenic and control animals were similar (and the same as Glut4-containing vesicles from rat adipocytes; data not shown) and that these structures in both cases were very efficiently separated from the bulk of total protein in the LM fraction. This strongly suggests that in transgenic animals, extra synthesized Glut4 does not spill into intracellular microsomes of a different size, but rather is compartmentalized in specific particles similar or identical to those from fat cells obtained from normal animals.

In the next experiment, we ran equilibrium density sucrose gradients to determine the buoyant density of Glut4-containing membrane structures. Fig. 3 (B and C) demonstrates that, as in the previous experiment, much more Glut4 is recovered when LM from transgenic mice were loaded on the gradient in comparison with the nontransgenic control mice. Moreover, Glut4-containing structures from transgenic animals have a higher buoyant density than Glut4-containing vesicles from normal mice, which is consistent with the possibility of more Glut4 molecules per vesicle. However, paradoxically, the distribution of the total protein was also shifted in these gradients to the...
Glut4 Targeting in Transgenic Mice

high density zone (Fig. 3A). To clarify these data, we assessed the density distribution of Glut1-containing vesicles, which are known to represent a different vesicle population than those containing Glut4 (Zorzano et al., 1989; Kandror et al., 1995a; this paper), and found out that the buoyant density of Glut1 vesicles was also increased (Fig. 3D), although there is no overexpression of Glut1 in the transgenic mice. Several other proteins abundant in LM, like SCAMPs and Rab4, also demonstrate a shift to the high density zone (data not shown). However, caveolin-containing vesicles retain the same buoyant density both in normal and transgenic adipocytes (Fig. 3E). This further proves that caveolin is not associated with Glut4 in the same vesicles (Kandror et al., 1995b) and suggests that the nature of caveolin-containing structures is different from that of Glut1- and Glut4-containing vesicles.

The direct experiment to compare Glut4-containing compartments from adipocytes of transgenic and normal mice is the comparison of their polypeptide composition. To isolate these structures, we applied the same experimental protocol that we use for immunoadsorption of Glut4-containing vesicles from rat adipocytes (Kandror and Pilch, 1994a), and this proved to be very efficient for mouse-derived vesicles as well. We were able to immunoabsorb ~90% of Glut4 from mouse LM with very low nonspecific adsorption (Fig. 4A). In agreement with previously published data, we immunoabsorbed only 2-3% of the total protein, which is consistent with the data illustrated in Fig. 2 and proves that Glut4-containing structures represent a specific minor population of total LM membranes even from adipocytes of transgenic animals. Interestingly enough, there was no Glut1 found in immunoabsorbed material (Fig. 4B), which once again demonstrates the existence of distinct vesicle populations for each of the transporters. This fact suggests that adipocytes have a high fidelity sorting mechanism that does not mix Glut1 and Glut4 even under conditions of considerable overexpression of Glut4. More important, the intracellular sorting of Glut4 to this distinct vesicle population is not saturated even at 15-20-fold overexpression.

By silver staining of the immunoabsorbed material (Fig. 5A), we have shown that Glut4-containing vesicles from transgenic and normal mice are composed of precisely the same constituent proteins. The only protein that appears different is Glut4 itself, which is the expected result. Western blotting with anti-SCAMP antibody (Thoidis et al., 1993) does not reveal any differences in the amount or electrophoretic mobility of these vesicle proteins (Fig. 5B). Unfortunately, our antibody against another marker protein for Glut4-containing vesicles, the aminopeptidase gp160, appeared to be rat-specific, and although we could recognize a major protein of 160 kDa on a silver-stained gel, we could not confirm its identity by Western blot analysis. Instead, we measured the enzymatic activity of gp160 in Triton X-100 eluates from Immunobeads and showed in three independent experiments that its activity was elevated only 1.3–1.5-fold in transgenic animals, which is still far from the increase in Glut4 content.

In this paper, we studied compartmentalization of Glut4 in adipocytes from transgenic mice overexpressing Glut4 at high levels selectively in adipose tissue (Shepherd et al., 1993). Under these conditions, Glut4 is found in the same type of vesicles as in normal cells according to protein composition and sedimentation analysis. We propose that in adipocytes from transgenic animals, there may be more Glut4 molecules packed into one vesicle than in control cells. An alternative explanation for our result is formation of a higher number of vesicles with normal Glut4 content. Since no drastic increase in any other vesicle protein component was detected, we think that the first explanation is more likely to be true.

Of course, the most direct way to prove this hypothesis would be to compare the buoyant density of Glut4-containing vesicles from normal and transgenic animals. This analysis was complicated by the unexpected result that the buoyant density of the total LM fraction is much higher in transgenic mice than in control mice. We do not have an explanation for this phenomenon yet. Since the total protein composition of the LM fraction does not seem to change (Fig. 1), it may be the phospholipid portion of the membrane that accounts for this very significant effect. We plan to perform a high pressure liquid chromatography-based analysis of the LM lipids to determine whether there are major alterations in the amount and/or composition of the lipids.

In general, transgenic adipocytes are quite similar to control cells and have very close physiological parameters such as their size (0.3 mg of lipid/cell for both nontransgenic and transgenic adipocytes used in this study). Elevated basal and insulin-stimulated glucose transport in transgenic adipocytes results in increased glucose metabolism at both rate-limiting
and physiological glucose concentrations with preferential effects on regulation of de novo fatty acid synthesis (Tozzo et al., 1995). A question may arise as to how the results described in this paper can be interpreted in light of the previous observation that basal glucose transport is elevated in transgenic adipocytes. In fact, these findings are consistent. It has been shown that under basal conditions, i.e. in the absence of insulin, Glut4 constantly recycles between its intracellular vesicular compartment and the plasma membrane (J hun et al., 1992; Yang et al., 1992; Czech and Buxton, 1993; Satoh et al., 1993; Yang and Holman, 1993) in such a way that ~2-5% of the transporter is present on the cell surface at any given moment of time.

Naturally, in transgenic animals expressing 15–20 times more Glut4, a dramatic increase in plasma membrane transporter would be expected in unstimulated adipocytes, which in turn would lead to a considerable elevation of basal glucose transport. Along with this, we cannot entirely rule out the possibility that some Glut4 may be also present in other structures that are more likely to target to the plasma membrane. To check this hypothesis, we tried to determine a potential presence of extra synthesized Glut4 in Glut1- and caveolin-containing vesicles. We considered that since there is more Glut1 and, especially, caveolin on the cell surface than inside the cell, the equilibrium between these intracellular vesicles and the plasma membrane must be shifted toward the latter compartment. However, in full agreement with previously published data (Zorzano et al., 1989; Kandror et al., 1995a, 1995b), we found no significant colocalization of these proteins with Glut4, neither by immunoadsorption (Glut1; Fig. 4) nor by sedimentation analysis (caveolin; Fig. 3).

As has been shown earlier (Shepherd et al., 1993), insulin-stimulated glucose transport is also increased severalfold in transgenic adipocytes in comparison with control normal cells. This is consistent with the results from our present study that overexpressed Glut4 is compartmentalized in specific vesicles that must be capable of delivering it to the cell surface in an insulin-dependent fashion. This suggests the following. 1) Adipocytes have a powerful intracellular machinery of precise targeting of Glut4 to specific vesicles. 2) These vesicles appear to be different from other intracellular microsomal structures since only they are selected as a target for Glut4 compartmentalization. 3) These vesicles appear to have a high reserve capacity to accept (and to translocate to the cell surface) much more Glut4 than under normal conditions. Another important conclusion is that overexpression of Glut4 in insulin-sensitive tissues, which appears to enhance both basal and insulin-stimulated glucose uptake, may lead to development of a genetic treatment for diabetes.

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