Comparison of glucose-transporter-containing vesicles from rat fat and muscle tissues: evidence for a unique endosomal compartment

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Insulin-sensitive tissues (fat and muscle) express a specific isoform of glucose-transporter protein, GLUT4, which normally resides in intracellular vesicular structures and is translocated to the cell surface in response to insulin. Here we provide a biochemical comparison of GLUT4-containing structures from fat and muscle cells. We demonstrate that, in spite of totally different protocols for cell homogenization and fractionation used for adipocytes as compared with skeletal-muscle tissue, GLUT4-containing vesicles from both sources have identical buoyant densities, sedimentation coefficients, and a very similar, if not identical, protein composition. The individual proteins first identified in GLUT4-containing vesicles from adipocytes (GTV3/SCAMPs proteins and aminopeptidase gp160) are also present in the analogous vesicles from muscle. Intracellular microsomes from rat adipocytes also contain GLUT1, a ubiquitously expressed glucose-transporter isoform. GLUT1 has not been detected in

intracellular vesicular pool(s) from skeletal-muscle cells, probably because of its low abundance there. GLUT1 in adipocytes is excluded from GLUT4-containing vesicles, but is found in membrane structures which are indistinguishable from the former by all methods tested and demonstrate the same type of regulation by insulin. That is, the GLUT1- and GLUT4-containing vesicles have identical densities and sedimentation coefficients in sucrose gradients, and translocate to the cell surface in response to hormonal exposure. Also, we describe a simple procedure for the purification of native glucose-transporter vesicles from rat adipocytes. Overall, our data suggest the existence of a unique endosomal compartment in fat and muscle cells which is functionally and compositionally different from other microsomal vesicles and which is responsible for insulin-sensitive glucose transport in these tissues.

INTRODUCTION

The post-prandial maintenance of blood glucose homeostasis is one of the most important physiological roles of insulin. It is achieved mainly via stimulation of glucose uptake in the insulin-sensitive tissues: fat, cardiac and skeletal muscle [1,2]. These tissues have a unique glucose-transporting machinery whose activity is up-regulated more than 10-fold by insulin. The central part of this machinery is a tissue-specific isoform of the glucose-transporter protein, GLUT4, which is expressed exclusively in fat and muscle cells. The hormone-dependent translocation of GLUT4 from an intracellular storage pool to the cell surface is the major, and most probably the only, mechanism of insulin action on glucose transport in fat and muscle under normal physiological conditions [3–6].

Most of the published studies concerning GLUT4-containing vesicles have been carried out on acutely isolated rat fat-cells or 3T3-L1 cultured murine adipocytes. It has been shown, however, that skeletal muscle is the predominant site for insulin-stimulated glucose disposal, whereas adipocytes contribute to a relatively minor degree in normal individuals [7], but fat can be a major glucose sink in very obese subjects [8]. GLUT4 trafficking in skeletal muscle is less well studied, mainly because of the difficulties in fractionation and isolation of membranes from muscle. In fat-cells, GLUT4 is found in membrane vesicles which are usually co-purified with a fraction of cellular light microsomes composed mainly of Golgi and the *trans*-Golgi network membrane structures [9]. A few studies have suggested that, in

muscles, GLUT4 is also present in microsomal vesicles related to the *trans*-Golgi tubulo-vesicular system [10–15]. Therefore, these specialized GLUT4-enriched membrane vesicles play a major role in the hormone-sensitive transportation of GLUT4 to its functional site at the cell surface, and they are the most likely site of insulin regulation. According to an alternative point of view, GLUT4 may utilize the same recycling pathway as plasma-membrane receptors do, sharing with them the same non-specialized components of the endosomal system (reviewed in [16]). To discriminate between these two possibilities, it will be important to characterize and to compare GLUT4-containing membrane structures which exist in different insulin-sensitive tissues.

The situation regarding glucose-transport regulation in fat and muscle tissues is complicated by the fact that, besides GLUT4, they express another isoform of glucose-transporter protein, GLUT1, which is not tissue-specific and can be found in different amounts in many cell types [16]. Under basal conditions, this isoform is present mainly at the cell surface and to a lesser extent in intracellular microsomes (at least, in adipocytes), and may also contribute to the insulin effect on glucose transport.

The aim of this study was to determine and to compare the main physico-chemical parameters of glucose-transporter-containing vesicular structures from insulin-sensitive tissues. We demonstrate that GLUT4-containing vesicles from fat and muscle cells have identical sedimentation coefficients and buoyant densities and a very similar polypeptide composition. The results obtained provide biochemical evidence that, regardless of the cell

type, GLUT4-containing vesicles may represent a unique specialized population of intracellular particles, functionally different from the other endosomal vesicles. The existence of such a 'mature storage organelle' is more consistent with the first, 'secretory', model of GLUT4 recycling, than with the 'receptor' model [16].

Intracellular GLUT1 in rat adipocytes is also found in vesicles which are translocated to the cell surface in response to insulin and have the same basic parameters as GLUT4-containing vesicles, but represent another vesicle population. Muscle cells do not seem to have the analogous GLUT1-containing intracellular membranes, or possibly their amount is below the detection limit of our experiments. On the basis of sedimentation behaviour and density distribution of glucose transporter-containing vesicles, a rapid and effective purification procedure has been developed. It gives a helpful alternative to vesicle immunoadsorption in cases where intact vesicles are required (direct glucose-transport assays, electron microscopy etc.).

MATERIALS AND METHODS

Antibodies

In the present study, we used a polyclonal antibody against GLUT1 (a gift from Dr. C. Carter-Su, University of Michigan), monoclonal 1F8 antibody against GLUT4 [3], monoclonal 3F8 antibody against GTV3/SCAMPs proteins [17], and whole anti-gp160 serum [18].

Adipocyte fractionation

Adipocytes were isolated from the epididymal fat-pads of male Sprague-Dawley rats (150–175 g) by collagenase digestion [19] and transferred to KRP buffer (12.5 mM Hepes, 120 mM NaCl, 6 mM KCl, 1.2 mM MgSO₄, 1 mM CaCl₂, 0.6 mM Na₂HPO₄, 0.4 mM NaH₂PO₄, 2.5 mM D-glucose, 2% BSA, pH 7.4) heated to 37 °C. Insulin was administered to cells (where indicated) to a final concentration of 10 nM for 15 min, then 0.2 M KCN was added for 5 min to a final concentration of 2 mM. Cells were washed three times with HES buffer (20 mM Hepes, 250 mM sucrose, 1 mM EDTA, 5 mM benzamidine, 1 mM phenylmethanesulphonyl fluoride, 1 µM pepstatin, 1 µM aprotinin, 1 µM leupeptin, pH 7.4) at 18 °C, homogenized with a Potter-Elvehjem Teflon pestle, and subcellular fractions were prepared as previously described [20]. For further fractionation by velocity or density-gradient centrifugation, the light-microsome fraction was pelleted on a 50%-sucrose cushion to avoid a resuspension step and transferred to PBS containing a standard cocktail of protease inhibitors by passing through a 5 ml Presto Column (Pierce).

Skeletal-muscle fractionation

Membrane fractions from skeletal muscle were isolated as described by Klip et al. [10]. Briefly, muscles were homogenized in a buffer containing 20 mM Hepes, 250 mM sucrose, 2 mM EDTA, 1 µM leupeptin, 1 µM pepstatin and 1 µM aprotinin A, pH 7.4 at 4 °C. The homogenate was centrifuged at 1200 g for 10 min at 4 °C, the supernatant was saved, and the pellet was resuspended, homogenized, and re-centrifuged at 1200 g for 10 min. The two supernatants were combined and centrifuged at 9000 g for 10 min at 4 °C. The resulting supernatant was then centrifuged at 200000 g for 90 min at 4 °C. The pellet was resuspended in 38% sucrose, and a discontinuous sucrose gradient consisting of 25%, 30%, 32% and 36% sucrose solutions (in 10 mM Hepes/1 mM EDTA, pH 7.4) were layered

over the membranes. The mixture was spun at 68000 g for 12–14 h at 4 °C. Light microsomes were isolated from the 32% sucrose fraction, diluted in homogenization buffer and centrifuged at 200000 g for 90 min. The resulting membranes were finally resuspended in PBS, pH 7.4.

Fractionation of intracellular microsomes in sucrose velocity gradients

Light microsomes from rat adipocytes and muscles, suspended in PBS, were loaded on a 4.6 ml 10–30% continuous sucrose gradient, or on a 28 ml 10–35% continuous sucrose gradient, and centrifuged at 48000 rev./min in a Beckman SW-50.1 rotor for 55 min or at 24000 rev./min in a Sorvall AH-627 rotor for 3.5 h respectively. All centrifugations were carried out at 4 °C. Buffer for both velocity and density sucrose gradients included 20 mM Hepes, 100 mM NaCl, 1 mM EDTA and 2 mM dithiothreitol, pH 7.5. In some cases, PBS was substituted for this buffer without any noticeable change in the results. Membranes from the gradients were collected into 25–30 fractions, starting from the bottom of the tubes. The protein concentration and refractive index were measured in samples. The position of glucose-transporter-containing vesicles was determined by Western blotting.

Fractionation of intracellular microsomes in sucrose density gradients

This was carried out in 4.6 ml 10–50% or 20–40% continuous sucrose gradients at 48000 rev./min in a Beckman SW-50.1 rotor for 16–18 h at 4 °C. After centrifugation, gradients were fractionated and analysed as described above.

Immunoadsorption of GLUT4 vesicles from fat and muscle microsomes

Protein A-purified 1F8 antibody, as well as non-specific mouse IgG (Sigma), were coupled to acrylic beads (Reacti-gel GF 2000, Pierce) at a concentration of 0.8–1.1 mg of antibody/ml of resin according to the manufacturers' instructions. Before use, the beads were saturated with 2% BSA in PBS for at least 1 h and washed with PBS. Light microsomes from rat tissues were incubated separately with each of the specific- and non-specific-antibody-coupled beads overnight at 4 °C. The beads were washed five times with PBS, and once with 10 mM Tris/HCl, pH 7.5, and the adsorbed material was eluted with Laemmli sample buffer without 2-mercaptoethanol.

Gel electrophoresis and Immunoblotting

Proteins were separated in SDS/polyacrylamide gels as described by Laemmli [21] and transferred to an Immobilon-P membrane (Millipore) in 25 mM Tris/192 mM glycine. After transfer, the membrane was blocked with 10% non-fat dry milk in PBS for 2 h at 37 °C. GLUT1, GLUT4, GTV3/SCAMPs and gp160 were detected with specific primary antibodies and ¹²⁵I-labelled or alkaline phosphatase (AP)- or horseradish-peroxidase (HRP)-conjugated secondary antibodies. A chemiluminescent substrate kit (NEN) was used in the last case.

Quantification of GLUT4 after Western blotting

Where ¹²⁵I-labelled secondary antibodies were used, after autoradiography, GLUT4-containing bands were cut from the membrane and counted in a γ -radiation counter. When HRP-conjugated secondary antibodies were used, the autoradiograph was scanned in a computing densitometer (Molecular Dynamics),

and results are expressed in arbitrary units. For some experiments both approaches were utilized, with virtually identical results.

Electron microscopy

Preparations of GLUT4-containing vesicles obtained by subsequent sucrose density and velocity gradient centrifugations were negatively stained with 0.5% uranyl acetate and analysed on a Hitachi EM 7000 electron microscope at 75 kV. Images were recorded at 20000–30000 $\times$ magnification.

Protein content

This was determined with a BCA kit (Pierce), according to the manufacturer's instructions.

RESULTS

Figure 1 represents the distribution of glucose transporters, GLUT1 and GLUT4, in subcellular fractions of rat adipocytes treated (+) and not treated (–) with insulin. When analysing these results, one should keep in mind that the signals produced by GLUT1 and GLUT4 on Western blots cannot be quantitatively compared, because of different antigen–antibody affinities and staining protocols. Quantitative comparisons of relative GLUT1 and GLUT4 amounts by affinity labelling [4–6] show that GLUT4 is 10–20 times more abundant than GLUT1 in rat adipocytes. Under basal conditions, fat-cell plasma membranes contain predominantly GLUT1, whereas the heavy and light intracellular microsomes have both GLUT1 and GLUT4. The small amount of GLUT1 present in a low-speed pellet of mitochondria and nuclei represents contamination of this fraction with plasma membrane. After insulin administration, both transporters are translocated to the plasma membrane from their intracellular pool(s). However, by the immunoadsorption of GLUT4-containing material on 1F8-beads, we demonstrated that GLUT4 and GLUT1 were present in different membrane populations (Figure 2; see also [5]), since > 90% of the GLUT1 was found in the supernatant with 97–98% of total light-microsome protein, whereas 2–3% of total light-microsome protein and 80% of GLUT4 were immunoadsorbed with specific antibody coupled to beads (Figure 2).

Further fractionation of light microsomes from rat adipocytes was carried out in velocity sucrose gradients, and the position of

Figure 1 Distribution of GLUT1 and GLUT4 in subcellular fractions of insulin-treated and untreated adipocytes

Portions (50 μ g of membrane protein) of the subcellular fractions indicated were electrophoresed and Western-blotted either for GLUT1 (detected with HRP-conjugated secondary antibodies and chemiluminescent substrate kit) or for GLUT4 (detected with 125 I-labelled secondary antibodies). Abbreviations for fractions are as follows: PM, plasma membrane; LM, light microsomes; HM, heavy microsomes; Cyt, cytosol; M/N, combined fraction of mitochondria and nuclei.

Figure 2 GLUT1 is excluded from GLUT4-containing vesicles

Light microsomes (0.53 mg of protein) isolated from insulin-treated and untreated rat adipocytes were immunoprecipitated with 100 μ l of 1F8-bound or non-specific IgG beads as described in the Materials and methods section. Eluted material was divided in half, electrophoresed and stained for GLUT1 and GLUT4 by using HRP-conjugated secondary antibodies and chemiluminescent substrate kit.

glucose-transporter-containing structures was determined by Western blotting with specific antibodies (Figure 3). The lower panel of Figure 3 shows the original autoradiograms (for GLUT4) or AP-stained membrane (for GLUT1), and quantification of total protein and GLUT4 distribution are presented as a graph on the upper panel. As is somewhat evident from the raw data of the bottom panel, the GLUT1 profile mirrored that of GLUT4, and the quantification is therefore not shown, to enhance clarity. The sucrose concentration was determined to be identical in both gradients (with or without insulin), as measured by refractive index (results not shown). The vertical arrow indicates the position of the sedimentation standard, 70 S ribosomes from *Escherichia coli*, centrifuged in a parallel gradient. The distribution of total microsomal protein (upper panel, squares) is not altered by insulin administration (results not shown). GLUT1- and GLUT4-containing structures have a rather narrow sedimentational distribution and are well separated from the other intracellular light microsomes (see also [22]), since they sediment faster than most of the light microsomes. We conclude that both transporters are localized in homogeneous membrane vesicles of similar sedimentation coefficients. After insulin administration, the amount of GLUT1- and GLUT4-containing vesicles present in intracellular light microsomes decreases substantially, due to their translocation and fusion with the plasma membranes (see also Figures 1 and 2). As shown in Figure 3 (black circles, upper panel), insulin also induces a small, but distinct, shift in the sedimentation coefficient of GLUT4-containing vesicles compared with untreated cells (white circles). Quantitative analysis of these data reveals that insulin produces a 15–20 S increase in sedimentation coefficient of GLUT4-containing vesicles in adipocytes.

Another basic parameter in the characterization of biological particles is their buoyant density. Figure 4 demonstrates the results of the centrifugation of light microsomes from adipocytes in a gradient spun to equilibrium. As in the previous experiment, the distribution of the GLUT1-containing vesicles was found to

Figure 3 Fractionation of light microsomes from rat adipocytes in a sucrose velocity gradient

Light microsomes (1.3 mg of membrane protein) from insulin-treated and untreated adipocytes were centrifuged in a 28 ml 10–35% sucrose gradient as described in the Materials and methods section. Lower panels show Western blot of odd-numbered gradient fractions, developed with ^{125}I -labelled (for GLUT4) or AP-conjugated (for GLUT1) secondary antibodies. Upper panel shows quantification of the GLUT4 distribution: ○, GLUT4 -insulin; ●, GLUT4 + insulin; □, total protein (only for adipocytes incubated without insulin). The vertical arrow is at the position of the 70 S *E. coli* ribosomes.

be identical with that of GLUT4, and so only the original AP-stained membrane is presented (lower panel). The distribution of GLUT4-containing vesicles (circles) was measured by computer densitometry (upper panel). As shown in the upper panel, GLUT4-containing vesicles from adipocytes have a buoyant density which more or less corresponds to the average density of the microsomal fraction (squares). Both GLUT4- and GLUT1-containing vesicles, however, are characterized by a rather narrow density distribution, which means that they have uniform and constant protein/lipid ratio. Insulin administration causes a certain shift in the position of GLUT4-containing vesicles, decreasing their density from 1.13 to 1.11 g/cm³ (determined by refractive index of sucrose solution in the corresponding zone with the correction for the buffer used). These slight, but nevertheless quite noticeable and very reproducible, alterations in sedimentation coefficient and buoyant density of GLUT4-containing vesicles may be indicative of insulin-induced changes in their structure and/or composition.

The ability of glucose-transporter-containing vesicles to be separated from other microsomes by gradient centrifugations gave us an opportunity to obtain highly purified preparations of vesicles by subsequent centrifugations in density and velocity sucrose gradients. For this purpose, light microsomes, freshly isolated from rat adipocytes, were fractionated overnight in a 10–50%-sucrose density gradient. GLUT4-containing fractions (identified by Western blotting) were pooled, diluted to 10% sucrose, and material was pelleted as described in the Materials and methods section. The second and the last purification step was a velocity centrifugation analogous to that described above,

Figure 4 Fractionation of light microsomes from rat adipocytes in a sucrose density gradient

Light microsomes (0.1 mg of membrane protein) from insulin-treated and untreated adipocytes were centrifuged in a 4.6 ml 20–40% sucrose gradient as described in the Materials and methods section. Various conditions are as in the legend to Figure 3, except for GLUT4 detection, which was carried out with the help of HRP-conjugated secondary antibodies and chemiluminescent substrate kit.

Figure 5 Electron microscopy of purified glucose-transporter-containing vesicles from rat adipocytes

after which GLUT4-containing fractions were again pooled, diluted and pelleted. Electron microscopy of this preparation (Figure 5) revealed a very homogeneous and uniform population of round-shaped vesicles with an average diameter of 50 nm, which is typical for GLUT4-containing vesicles [14,22]. The

Table 1 Purification of glucose-transporter-containing vesicles from light microsomes (LM) of rat adipocytes

GLUT4 content was estimated by quantitative Western blotting of 10 μ l samples of each fraction, using 125 I-labelled secondary antibodies; the LM value was set to 100%.

Fraction	Protein (mg)	GLUT4 yield (%)	Purification (fold)
LM	2.0	100	1
Density gradient	0.22	100	9
Velocity gradient	0.030	64	42.7

Figure 6 Fractionation of light microsomes from rat skeletal muscle in sucrose velocity gradient

Skeletal-muscle light microsomes (0.2 mg of membrane protein) from rats injected with insulin or not were centrifuged in a 4.6 ml 10–30% sucrose gradient as described in the Materials and methods section. For designations, see the legend to Figure 3. GLUT4 was detected with 125 I-labelled secondary antibodies (upper panel) or with HRP-conjugated secondary antibodies and chemiluminescent substrate kit (lower panel).

vesicles apparently had not been destroyed during isolation, since there was no penetration of stain inside them. The specific content of GLUT4 in the purified preparation as compared with total light microsomes was increased more than 40-fold according to quantitative Western blotting carried out with 125 I-labelled secondary antibodies (Table 1). In other words, GLUT4-containing vesicles purified in this fashion represent 2.5% of the total light-microsome protein, a result in excellent agreement with the estimation obtained by cytochalasin B binding and immunoadsorption [5,22]. Light microsomes comprise 8–9% of protein recovered in all subcellular fractions taken together (plasma membranes, heavy microsomes, light microsomes, cytosol, mitochondria and nuclei), so GLUT4-containing vesicles represent about 0.2% of total adipocyte protein. Our preparation of purified vesicles should include both GLUT4- and GLUT1-containing vesicles, which are indistinguishable by all methods applied. However, the amount of GLUT4-containing vesicles is

Figure 7 Fractionation of light microsomes from rat skeletal muscle in sucrose density gradient

Skeletal-muscle light microsomes (0.14 mg of protein) from rats injected with insulin and from control rats was centrifuged in a 4.6 ml 20–40% sucrose gradient as described in the Materials and methods section. For designations see the legend to Figure 3. GLUT4 was detected with HRP-conjugated secondary antibodies and chemiluminescent substrate kit.

much higher than that of GLUT1-containing vesicles (see above), so the latter do not interfere with our calculations.

Our further work was directed to characterization of GLUT4-containing vesicles from rat skeletal muscle, the main tissue responsible for insulin-regulatable glucose uptake *in vivo*. Figures 6 and 7 demonstrate that, in spite of a completely different distribution of total microsomal protein from fat and muscle tissues, the size and total protein/lipid composition (i.e. buoyant density) of GLUT4-containing vesicles are virtually identical on the basis of their sedimentation coefficients and densities in sucrose solutions. The position of the *E. coli* ribosomal marker in Figures 3 and 6 is identical with respect to the GLUT4 distribution profiles in these velocity gradients. Similarly, the density of the GLUT4 vesicles centres between fractions 15 and 20 for both fat- and muscle-derived membranes. As is the case for isolated adipocytes treated with insulin, injection of this hormone into rats induces the expected decrease in the amount of intracellular GLUT4-containing vesicles, due to their fusion with plasma membrane, and also causes a shift in their sedimentation coefficient and buoyant density. These changes, however, were less pronounced in the case of muscle, since the normal physiological level of insulin in the rats' blood should cause partial translocation of GLUT4-containing vesicles to some degree. Adipocytes, on the other hand, were preincubated in an insulin-free medium before administration of the hormone. We were unable to observe intracellular GLUT1-containing vesicles in rat muscle cells, most probably because their amount is below our detection limit.

In agreement with the similarities in sedimentation coefficients and buoyant densities of GLUT4-containing vesicles from fat and muscle cells, immunoisolation of these structures on 1F8 antibody covalently bound to acrylic beads demonstrated that

Figure 8 Polypeptide composition of GLUT4-containing vesicles from rat adipocytes and muscle

Light microsomes (0.5 mg of membrane protein) from rat adipocytes and muscles were immunoprecipitated with 100 μ l of 1F8-bound or non-specific IgG beads as described in the Materials and methods section. The Figure represents silver staining of a 9%-polyacrylamide gel, with molecular-mass standards (kDa) shown on the right.

their total polypeptide composition is rather similar (Figure 8). With the amount of protein loaded on these gels, it is hard to detect GLUT4 by silver staining because of its poor staining properties, probably due to its dispersed migration pattern [5,14]. However, a few of the major vesicle constituents, such as three

high-molecular-mass glycoproteins, gp110, gp160 and gp230 [23], are present in the preparations of GLUT4-containing vesicles from both fat (lane 1) and muscle (lane 4). To confirm this result, Western-blot analysis was carried out, using specific antibodies against some of the individual vesicle proteins known to be present in rat adipocytes. Shown in Figure 9(a) is a blot of gp160, a membrane glycoprotein possessing aminopeptidase activity [24], which is completely co-localized with GLUT4-containing vesicles from adipocytes. This protein represents one of the major vesicle components, and may be considered as a marker protein of GLUT4-containing vesicles [18,23,25]. Another recently identified vesicle constituent from adipocytes is a triplet of GTV3, or SCAMPs (secretory carrier-associated membrane proteins), whose function is unknown, but whose widespread distribution in intracellular membranes and trafficking to the cell surface suggests that they may play an important role in vesicle fusion with the plasma membrane [17,26]. The presence of these proteins in analogous vesicles from muscles would indicate their general importance for the organization of insulin-sensitive glucose-transporting machinery regardless of the cell type. Although the level of non-specific adsorption of primary and secondary antibodies during Western blot analysis of muscle samples is very high, Figure 9(b) clearly demonstrates that gp160 and GTV3 are specifically present in GLUT4-containing vesicles isolated from rat skeletal muscle, and could not be detected in the material non-specifically adsorbed on total mouse IgG.

DISCUSSION

In this study we provided a comparative biochemical study of GLUT4-containing vesicles from the intracellular microsomes of fat and muscle cells. In the latter case we used a slightly modified procedure for muscle-cell fractionation, originally proposed by Klip et al. [10]. According to this procedure, GLUT4 recovery in the so-called fraction of intracellular light microsomes (used for further analysis) is about 60%, and all GLUT4 in this fraction is found in the form of GLUT4-containing vesicles, the detailed characterization of which was one of the purposes of this study.

Figure 9 gp160 and GTV3/SCAMPs proteins are present in GLUT4-containing vesicles from fat and muscles

A Western blot of the same preparation as in Figure 8 is shown. (a) Protein eluted from 1F8-bound beads and control non-specific immunobeads was separated in a 7.5%-polyacrylamide gel and Western-blotted with anti-gp160 antibody. (b) Preparations from muscle were electrophoresed in a 5–15% gradient polyacrylamide gel, whereas fat proteins were separated in a 10%-acrylamide gel; both were blotted with anti-GLUT4 1F8 antibody together with anti-GTV3/SCAMPs 3F8 antibody.

Other GLUT4 molecules, not involved in the light-microsomal fraction, are present in the other subcellular structures of skeletal-muscle cells, for example, in the t-tubules [27]. On the contrary, in fat-cells under basal conditions, practically all the GLUT4 accessible for biochemical analysis resides in the population of the uniform vesicles. We have demonstrated that GLUT4-containing vesicles from fat and muscle cells have an essentially identical and narrow sedimentation and density distribution. The buoyant density of these particles determined in an equilibrium sucrose gradient is 1.11–1.13 g/cm³, depending on whether or not the cells were first exposed to insulin. This number is very close to the buoyant density of GLUT4-containing vesicles from 3T3-L1 adipocytes [28], although the protein composition of these vesicles in 3T3-L1 adipocytes and in native rat adipocytes may be somewhat different. For example, the latter do not include GLUT1 and insulin-like growth factor-II receptor (Figure 3, and [5]), whereas in 3T3-L1 adipocytes it has been reported that these proteins are co-localized with GLUT4 in the same vesicles [29,30]. In 3T3-L1 cells, the GLUT4:GLUT1 ratio (although somewhat different in different clones) is about 1:3, whereas in rat adipocytes it is at least 10:1, and thus the much greater amount of GLUT1 in the former cells may underlie the reason for this apparent discrepancy.

The results of these experiments demonstrate that GLUT4-containing structures represent a homogeneous vesicle population which behaves like individual particles and has a distinct and constant protein composition. These vesicles are most probably not originating from other subcellular structures such as tubules and cisternae of the Golgi, or larger endosomes as a result of homogenization. Firstly, they are too uniform to be considered a degradation product; secondly, totally different homogenization procedures applied to fat and muscle cells give similar type of GLUT4-containing structures; thirdly, their properties change after insulin treatment of cells in an identical fashion. Thus GLUT4-containing vesicles are likely to be different from the other intracellular endosomes, not only functionally, but structurally as well. This conclusion is in total agreement with the results of gel-filtration as well as electron-microscopy analysis of partially purified GLUT4-containing vesicles [14,22], and sections of rat adipocytes [31]. Electron microscopy of brown adipose tissue and cardiac myocytes demonstrated more heterogeneity of GLUT4-containing structures than we observe [12,13]. Although most of the GLUT4 molecules in these studies were associated with more or less homogeneous vesicles, a visible part of the transporter was found in tubules and cisternae of the *trans*-Golgi network. The latter structures, however, may contain nascent molecules of GLUT4, which may be in the process of biosynthesis, glycosylation or sorting, waiting to be assembled with other proteins in a mature secretory vesicle. In cells overexpressing GLUT4, for example, it is found in virtually all membrane structures, including plasma membranes [32].

It has been shown that GLUT4-containing vesicles constantly recycle between an intracellular compartment and the plasma membrane in both the absence and the presence of insulin [33–36]. At present, controversial data exist as to whether insulin stimulates externalization and inhibits internalization [33,35], or just accelerates the exocytic step [36,37]. We were interested, however, in determining whether insulin-induced changes in GLUT4 trafficking were accompanied by structural alterations of the corresponding vesicles. Our data give the first evidence that insulin administration to rat adipocytes causes certain molecular reorganizations in GLUT4-containing vesicles, resulting in alterations of their sedimentation coefficient and buoyant density. These changes may reflect differences in the

protein/lipid ratio (as first noticed by Brown et al. [29]) or a change in the internal volume of GLUT4-containing vesicles. On the other hand, the observed differences in physico-chemical parameters of vesicles may be the consequence of their interaction with certain mediators, such as the 20 S particle originally discovered in Rothman's laboratory as a key component of the fusion of transport vesicles with Golgi membranes (for review see [38]), a process which is now thought to be conserved from yeast to humans [39]. This 20 S particle simultaneously binds receptor proteins (SNAREs) located in the vesicle membrane and in the target membrane, thus providing the first step of membrane fusion [40]. A candidate for the role of vesicle SNARE, VAMP or synaptobrevin, has recently been shown to be a component of GLUT4-containing vesicles [41].

Our data support the notion that glucose-transporter-containing vesicles represent a unique specialized population of intracellular endosomes, conserved in insulin-sensitive fat and muscle tissues. It will be interesting to compare the overall structure and polypeptide composition of these vesicles and some other types of specialized secretory vesicles, such as water-channel-containing vesicles, which are translocated to the apical surface of the collecting duct in response to antidiuretic hormone [42], a model system which may have much in common with the insulin-sensitive translocation of GLUT4-containing vesicles. The fact that both types of vesicles have a high level of intrinsic aminopeptidase N activity [24,43] emphasizes their possible structural similarity. The comparison of glucose-transporter-containing vesicles with other structures, such as the chromaffin granules of the adrenal medulla, neuronal synaptic vesicles, or zymogen granules of exocrine pancreas, may be also very useful for a better understanding of the structure of secretory machinery in different cell types.

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