Isolation and Characterization of the Two Major Intracellular Glut4 Storage Compartments*

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In rat adipose cells, intracellular Glut4 resides in two distinct vesicular populations one of which contains cellugyrin whereas another lacks this protein (Kupriyanova, T. A., and Kandror, K. V. (2000) J. Biol. Chem. 275, 36263–36268). Cell surface biotinylated MPR and 125I-labeled transferrin are accumulated in cellugyrin-positive vesicles and to a lesser extent in cellugyrin-negative vesicles. An average cellugyrin-positive vesicle carries not more than one molecule of either Glut4, insulin-responsive aminopeptidase (IRAP), or transferrin receptor (TfR), whereas cellugyrin-negative vesicles contain five to six molecules of Glut4, more than 10 molecules of IRAP, and still one molecule of TfR per vesicle. Cellugyrin-negative vesicles are translocated to the cell surface after insulin stimulation, whereas cellugyrin-positive vesicles maintain intracellular localization both in the absence and in the presence of insulin and, therefore, may be involved in interendosomal protein transport. Both cellugyrin-positive and cellugyrin-negative vesicles are present in extracts of non-homogenized cells and therefore may represent the major form of Glut4 storage in vivo.

In adipose and skeletal muscle cells, protein traffic between the cell surface and intracellular organelles is regulated by insulin (1). In particular, insulin causes dramatic redistribution of glucose transporter isofrom 4 (Glut4) and insulin-responsive aminopeptidase (IRAP)1/placental leucine aminopeptidase from intracellular compartment(s) to the plasma membrane. Recent studies have shown that in adipocytes, Glut4 and IRAP are not targeted to the regulated secretory pathway (2–5) but, rather, to the endosomal system. In adipocytes, other recycling endosomal proteins, such as the transferrin receptor (TfR) and the IGFII/mannose 6-phosphate receptor (MPR), demonstrate the same response to insulin as Glut4 and IRAP but to a significantly lesser degree (6–9). It has been suggested, therefore, that Glut4 and IRAP are sequenced into specialized post-endosomal insulin-sensitive vesicles, or IRVs with much higher insulin responsiveness than endosomes and non-specialized endosome-derived transport vesicles (10–12).

Although there is little doubt that some Glut4 is indeed localized in endosomes (13–19), a post-endosomal insulin-responsive compartment has been postulated but never isolated and characterized. This may be attributed to significant technical difficulties in the biochemical separation of the intracellular Glut4-containing membranes. In addition, it is not clear to what extent cell homogenization contributes to heterogeneity of intracellular “Glut4-containing vesicles” since large membrane structures may be artificially fragmented into smaller vesicles upon cell homogenization. So, at present, it is not known whether a translocatable pool of Glut4 is accumulated in small insulin-responsive vesicles or in a large endocytic recycling compartment. Correspondingly, it is not yet clear whether insulin stimulates translocation of pre-existing vesicles, stimulates budding of new vesicles from endosomes, or both.

Recently, we identified a novel protein, cellugyrin, in intracellular Glut4-containing membranes and prepared a monoclonal antibody against this protein (20). Cellugyrin, a four-transmembrane protein, represents a homologue of synaptogyrin, a major constituent of synaptic vesicles (21). In adipose cells, cellugyrin is co-localized with Glut4 (20). However, it is present only in the population of Glut4 vesicles, which is not recruited to the plasma membrane by insulin and permanently maintains its intracellular localization. Glut4 vesicles capable of cell surface translocation do not contain this protein (20).

Here, we demonstrate that cellugyrin-positive Glut4 vesicles rapidly accumulate cell surface biotinylated MPR and can be readily loaded with radioactive transferrin. We suggest, therefore, that these vesicles originate from early endosomes. Cellugyrin-negative vesicles contain significantly less of both biotinylated MPR and radioactive transferrin and show dramatic insulin-stimulated translocation to the cell surface. Thus, cellugyrin-negative Glut4 vesicles are likely to represent the long sought post-endosomal IRVs. We found that an average cellugyrin-positive vesicle carries not more than one molecule of either Glut4, IRAP, or TfR, whereas cellugyrin-negative IRVs compartmentalize five to six molecules of Glut4, more than 10 molecules of IRAP, and still one molecule of TfR per vesicle. Thus, there is a stringent sorting step in the “Glut4 pathway” that results in the specific enrichment of Glut4 and IRAP, but not TfR in the specialized insulin-responsive compartment. Finally, we describe an experimental approach that allows us to obtain adipocyte extracts without cell homogenization. Under these conditions, the major fraction of Glut4 is still found in small cellugyrin-positive and cellugyrin-negative vesicles. We suggest that these vesicles may thus represent the major compartments of Glut4 storage and translocation in the living cell.

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‡ The abbreviations used are: IRAP, insulin-responsive aminopeptidase; TfR, transferrin receptor; MPR, mannose 6-phosphate receptor; IRV, insulin-responsive vesicles; PBS, phosphate-buffered saline; LM, light microsomes; ER, endoplasmic reticulum.
Materials—In the present study, we used monoclonal anti-Glut4 antibody 1F8 (22). Anti-IRAP rabbit serum used for Western blots was a kind gift of Dr. P. Pilch, Boston University School of Medicine. Whole rabbit serum against the cytoplasmic tail of IRAP used for antibody decoration experiments was a kind gift of Dr. Lynn Kozma, Metabolex Inc., Hayward, CA. DEAB-cellulose-purified anti-IGF-II/IR antibody was a kind gift of Dr. M. Czech, University of Massachusetts Medical School. Rabbit anti-serum against sortilin was prepared by Quality Controlled Biochemicals, Inc., Hopkinta, MA using the peptide ac-CFGQSKLYSEDYKGNFKD-amide (amino acids 17–34) as antigen. Monoclonal anti-TfR antibody was from Zymed Laboratories Inc. Monoclonal anti-cellugyrin antibody was described previously (20).

Isolation and Fractionation of Rat Adipocytes—Adipocytes were isolated from the epididymal fat pads of male Sprague-Dawley rats (150–175 g) by collagenase digestion and transferred to KRP buffer (125 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 glucose, 2 mM bovine serum albumin, pH 7.4). Insulin was administered to cells (where indicated) to a final concentration of 10 nM for 15 min. After that, KCN was added to cells to final concentration 2 mM for 5 min. Cells were washed three to four times with HES buffer cooled to 14–16 °C (20 mM HEPES, 250 mM sucrose, 1 mM EDTA, 5 mM benzamidine, 1 mM phenylmethanesulfonyl fluoride, 1 μM pepstatin, 1 μM aprotinin, 1 μM leupeptin, pH 7.4), and homogenized with a Potter-Elvehjem Teflon pestle. Subcellular fractions were prepared as previously described (23). Isolated fractions were prepared by centrifugation, which contained all of the protease inhibitors listed above. Alternatively, non-homogenized cells were washed with HES and centrifuged at 16,000 × g for 20 min, which represents the first step of differential centrifugation in (23).

Internalization of 125I-Transferrin—125I-transferrin (PerkinElmer Life Sciences) was added to rat adipocytes to 1 μCi per ml of packed cells alone or together with 75 nM non-radioactive transferrin (Sigma). Cells were then treated with 2 mM KCN for 2 min at room temperature, washed, and homogenized as described above.

Immunoadsorption of Glut4- and Cellugyrin-containing Vesicles—Protein A-purified 1F8 antibody, anti-cellugyrin antibody, and nonspecific mouse IgG were each coupled to acrylic beads (Reacti-gel GF 2000, Pierce) according to the manufacturer’s instructions. Before use, the beads were saturated with 2% bovine serum albumin in PBS for at least 1 h and washed with PBS. The light microsomes (LM) from rat adipocytes were incubated separately with each of the specific and nonspecific antibody-coupled beads overnight at 4 °C. The beads were washed five times with PBS, and the adsorbed material was subsequently eluted with 1% Triton X-100 in PBS and Laemmli sample buffer without reducing agents in order to avoid dissociation of the coupled antibodies. The protein composition and insulin responsiveness of cellugyrin-positive and -negative vesicles were not able to reproduce an earlier report that showed the presence of mannose 6-phosphate/IGFII and transferrin as well as Glut4 and IRAP in localized cellugyrin-positive vesicles (26). In basolateral adipocytes, cellugyrin-negative vesicles compartmentalize significant fractions of Glut4 and IRAP (30–50%) and small, but readily detectable amounts of MPR and TR. Upon insulin administration, the amount of all recycling proteins is decreased in both cellugyrin-positive and -negative vesicles. The latter compartment, however, demonstrates a much more pronounced insulin response. These results along with previously published data (20) are consistent with the hypothesis that the cellugyrin-negative compartment represents early/sorting endosome-derived vesicles. However, we were unable to detect endosomal marker proteins, such as rab4, rab11, or EEA1, in either cellugyrin-positive or -negative vesicles. We suggest, therefore, that these peripheral membrane proteins dissociate from membranes upon vesicle formation from endosomes. It is also possible that these proteins are removed from immunoadsorbed vesicles by several stringent washes required for sufficient purity. In any case, we were not able to reproduce an earlier report that showed the presence of rab4 in immunopurified Glut4 vesicles (27).

To determine the nature of cellugyrin-positive and cellugyrin-negative Glut4 vesicles, we studied internalization of 125I-transferrin into insulin-treated and not treated adipocytes. We have shown earlier that about 50% of the internalized transferrin is recovered in Glut4 vesicles (28). We now confirm this result and demonstrate that, after 15 min of incubation with 125I-transferrin, practically all radioactivity in Glut4 vesicles is recovered in the cellugyrin-positive Glut4 population (Fig. 2). Addition of excess cold transferrin (75 nM) decreased the amount of internalized radioactivity to background (not shown). This result suggests that cellugyrin-positive vesicles account for virtually all early endosomal material in the total population of Glut4-containing membranes. To determine whether or not recycling receptors can be delivered to a cellugyrin-negative Glut4-containing compartment, we carried out two types of experiments. First, we studied the distribution of the cell surface-biotinylated MPR.
between cellugyrin-positive and cellugyrin negative Glut4 vesicles (Fig. 3A). We found that this receptor is rapidly internalized into cellugyrin-positive vesicles and only 30 min later does it become detectable in the cellugyrin-negative vesicles. Also, }^{125}\text{I}-\text{ transferrin is internalized mostly into cellugyrin-positive vesicles but is also detectable in cellugyrin-negative vesicles after prolonged incubations (Fig. 3B). We believe that these results are consistent with Fig. 1 and with our previous observations (25, 28, 29) showing that a certain fraction of TIR and MPR are present in both cellugyrin-positive endosomes and cellugyrin-negative post-endosomal IRVs. To more accurately compare the protein composition of these compartments, we used the following approach that we call an “antibody decoration technique”. The intracellular membrane fraction that contains the mixture of cellugyrin-positive and cellugyrin-negative Glut4 vesicles is incubated with nonspecific IgG or with antibodies against the cytoplasmic domains of Glut4, IRAP, or TIR. This material is fractionated in a velocity sucrose gradient, and the position of cellugyrin-positive vesicles is determined by Western blotting of the gradient fractions with anti-cellugyrin antibody (Fig. 4A). In a parallel experiment, the cellugyrin-positive compartment is removed from the membrane preparation by immunoadsorption as shown in Fig. 1, and cellugyrin-negative Glut4 vesicles are analyzed by the same technique. This time, however, anti-Glut4 antibody is used for Western blotting (Fig. 4B).

Binding of antibodies to vesicles makes them heavier and causes a shift in the gradient. This shift is proportional to the number of antibody molecules bound to the vesicle. Knowing that the sedimentation coefficient of the IgG molecule is 4 S, it is possible to estimate the average number of Glut4, IRAP, and TIR molecules in cellugyrin-positive and -negative vesicles.

Using this approach, we have shown that, although Glut4, IRAP, and TIR are certainly present in cellugyrin-positive vesicles (Fig. 1 and Ref. 20)), binding of specific antibodies does not induce significant shifts in the sedimentation coefficient of this compartment (Fig. 4A). This means that cellugyrin-positive vesicles contain not more than one molecule of either Glut4, IRAP, or TIR per vesicle (a shift of 5 S is readily detectable under these conditions). The same results were obtained when we used anti-cellugyrin and anti-VAMP2 antibodies for these experiments (results not shown). On the contrary, an average post-endosomal cellugyrin-negative Glut4 vesicle (IRV) carries approximately five to six Glut4 molecules (shift 22 S), more than 10 molecules of IRAP (shift >40 S), and still not more than one TIR molecule (Fig. 4B). Thus, Glut4 and IRAP, but not TIR, are specifically accumulated in cellugyrin-negative vesicles. These data clearly illustrate that there is a sorting step in the Glut4 pathway, which functions to specifically “gather” Glut4 and IRAP, but not TIR, and to pack them into post-endosomal vesicles, IRVs. In these experiments, we were not able to analyze cellugyrin-negative vesicles from insulin-treated cells, since their amount is very low, most likely, to almost complete fusion with the plasma membrane upon insulin stimulation.

In control experiments, we incubated LM with 1F8 antibody for different times (1, 2, and 5 h) and also used different amounts of 1F8 (5 and 25 µg) for the same amount of LM (150 µg). In all these cases, we obtained similar results (not shown). Thus, the shift in the sedimentational distribution of Glut4 vesicles caused by binding to 1F8 does not depend on experimental conditions.

Our data described here, as well as in our previous paper (20), indicate that the cellugyrin-positive Glut4-compartment represent intracellular transport vesicles that originate from early endosomes and cellugyrin-negative Glut4-compartment may be the IRVs. As it is seen in Fig. 4, A and B, the sedimentation coefficient of cellugyrin-positive vesicles is somewhat smaller than that of cellugyrin-negative vesicles (see also Ref. 20). This suggests that cellugyrin-positive vesicles do not represent intact endosomes, since these organelles are known to be large structures that should have a much higher sedimentation coefficient than small membrane vesicles (30, 31). It is quite possible, however, that upon cell homogenization endosomes are artificially fragmented into small vesicles of roughly the same size as vesicles formed in vivo.

To determine whether or not cellugyrin-positive and -negative vesicles represent an artifact of cell homogenization, we omitted the homogenization step during subcellular fractionation of rat adipocytes. It turns out that traditional homogenization with a Potter-Elvehjem homogenizer is absolutely not necessary for further manipulations of adipose cells. In the experiment shown in Fig. 5, the cell homogenate (panel A) or not homogenized, washed adipocytes (panel B) were placed in centrifuged tubes and centrifuged at 16,000 g for 20 min (the
first step of the “classical” protocol of Simpson et al. Ref. 23). Such centrifugation appears to be quite sufficient to break open primary adipocytes. The crude fractions resulting from the centrifugation (the lipid cake, the liquid phase, and the pellet) look identical regardless of whether or not the cells have been homogenized. The total amount of protein (the liquid phase and the pellet together) obtained was 2042 g per rat for homogenized and 2003 g per rat for non-homogenized cells. In both cases, the pellet accounts for 20–25% of the total protein recovered. Note, that presence of intact cells in the pellet is highly unlikely, since primary rat adipocytes float upon centrifugation.

As it was shown previously for the standard protocol (23) and confirmed in numerous laboratories including ours, the pellet of this centrifugation (the lipid cake, the liquid phase, and the pellet) look identical regardless of whether or not the cells have been homogenized. The total amount of protein (the liquid phase and the pellet together) obtained was 2042 ± 250.4 μg per rat for homogenized and 2003 ± 34 μg per rat for non-homogenized cells. In both cases, the pellet accounts for 20–25% of the total protein recovered. Note, that presence of intact cells in the pellet is highly unlikely, since primary rat adipocytes float upon centrifugation.

As it was shown previously for the standard protocol (23) and confirmed in numerous laboratories including ours, the pellet of this centrifugation is composed of heavy subcellular structures, such as nuclei, mitochondria, lysosomes, the plasma membrane, etc. Microscopic evaluation showed that all of these organelles were also present in the first pellet of our procedure (results not shown). However, as is seen in Fig. 5, homogenization dramatically changes the distribution of fragile subcellular structures, such as the endoplasmic reticulum and intermediate filaments, between the pellet and the supernatant. In non-homogenized cells, both ER and intermediate filaments were found almost exclusively in the pellet, while homogenization lead to visible fragmentation of these structures into smaller particles that could not be pelleted at 16,000 × g.

Cell homogenization did not significantly affect the distribution of Glut4, cellulagryrin, and the endosomal markers between the pellet and in the supernatant of the 16,000 × g centrifugation (Fig. 5). We thus suggest that, unlike endoplasmic reticulum and intermediate filaments, endosomes are not likely to be fragmented by homogenization and are actually pelleted under these conditions along with the plasma membrane, lysosomes, and other heavy intracellular organelles. This hypothesis is consistent with the sedimentational properties of endosomes obtained from homogenized PC12 cells (32) and Chines hamster ovary (33) cells.

In the following experiment we compared the sedimentational distribution of Glut4- and cellulagryrin-containing membranes obtained in the standard subcellular fractionation protocol (23) with those isolated from not homogenized cells. For that, we centrifuged LM (200 μg) along with the supernatant of the low-speed centrifugation (2 mg) shown in Fig. 5B in a continuous 10–30% sucrose gradient. For these experiments, we used 10 times more of the supernatant than pelleted LM, because 90% of the total protein in the supernatant is ac-

![Diagram](http://www.jbc.org/Downloadedfrom)
counted for by cytoplasm that does not contain proteins of interest. Note that unlike LM, membranes in the supernatant have never been pelleted and resuspended before gradient centrifugation. Nonetheless, Fig. 6 demonstrates that the distribution of Glut4, cellugyrin, IRAP, sortilin, TfR, and MPR is identical regardless of the protocol used for membrane isolation. On the contrary, fragments of ER and intermediate filaments were present only in LM, probably due to artificial fragmentation during homogenization. These structures were completely absent from the material prepared from non-homogenized cells. Thus, cell homogenization results in the artificial fragmentation of ER and cytoskeleton, but does not affect the size of cellugyrin-positive and cellugyrin-negative Glut4 vesicles.

**DISCUSSION**

We show here that the intracellular Glut4-containing membranes can be separated into two distinct compartments: cellugyrin-positive and cellugyrin-negative Glut4 vesicles. The first compartment has low specific content of recycling proteins and moderate insulin sensitivity. On the contrary, cellugyrin-negative vesicles have high specific content of Glut4 and IRAP and show a profound response to insulin (Fig. 1). Moreover, we present evidence that both types of vesicles are present in extracts of non-homogenized cells and, therefore, may represent the major Glut4 compartments in vivo. Such a conclusion is consistent with the results of electron microscopy demonstrating that in adipocytes, Glut4 resides in vesicles and short tubules rather than in typical endosomes (13, 16). Tubulovesicular Glut4-containing structures revealed by electron microscopy are 50–80 nm in diameter (13, 16). This size corresponds very well to the sedimentation coefficient of Glut4 vesicles determined in our experiments (50–80 S, Fig. 4). Thus, small Glut4 vesicles recovered in vitro upon subcellular fractionation may also exist in vivo. This indicates that the translocatable pool of Glut4 is retained in adipocytes in a form of small vesicles and not in a large endocytic recycling compartment. Based on this conclusion, we suggest that the primary effect of insulin should be translocation of pre-existing vesicles, rather than stimulation of the budding reaction, although these effects are not incompatible (see below).

Cellugyrin-positive vesicles carry both plasma membrane markers, such as 125I-transferrin-bound TfR and biotinylated MPR, and cellugyrin, a protein that, according to our previous studies, is not present at the plasma membrane (20). We think, therefore, that this compartment represents early endosome-derived vesicles. This explanation is totally consistent with the recent results of Lim et al., who showed that in Chines hamster ovary cells, endosome-derived small vesicles represent a major cellular pool for recycling membrane proteins including ectopically expressed Glut4 and endogenous IRAP (33). We suggest, furthermore, that cellugyrin-positive vesicles carry recycling proteins from early endosomes to recycling endosomes that function as a major sorting station in the cell (31, 34). It was shown previously that transition from early to recycling endosomes represents a default pathway that does not require any specific targeting sequences (35). In agreement with these data, we found that several recycling proteins, such as Glut4, IRAP, and TfR, are represented in cellugyrin-positive transport vesicles in a random fashion. Sorting in recycling endosomes by an as yet unknown mechanism results in the formation of

**FIG. 5.** Homogenization of primary rat adipocytes leads to shattering of the endoplasmic reticulum and intermediate filaments, but not endosomes. Homogenized (A) or non-homogenized (B) cells were centrifuged at 16,000 × g for 20 min at 4 °C. Aliquots of resulting pellets and supernatants (both 150 μg) were analyzed by Western blot.

**FIG. 6.** Homogenization does not change the sedimentational distribution of cellugyrin-positive and cellugyrin-negative Glut4 vesicles. LM (200 μg) obtained by standard procedure (23) or 16,000 × g supernatant from non-homogenized adipocytes (2 mg) were fractionated in 10–30% continuous sucrose gradients for 55 min at 48,000 rpm in a Beckman SW-50.1 rotor at 4 °C. Each gradient was separated into 23 fractions that were analyzed by Western blotting.
insulin-responsive cellugyrin-negative Glut4 vesicles or IRVs with much a higher content of specific recycling proteins such as Glut4 and IRAP. Cellugyrin is not allowed entry into IRVs and is probably returned back to early endosomes by rapid retrograde transport (36).

Cellugyrin-positive and cellugyrin-negative Glut4 vesicles demonstrate different responses to insulin stimulation. There is a small but consistent insulin-induced decrease in the amount of recycling proteins in cellugyrin-positive vesicles (Fig. 1). We think that insulin may stimulate to some degree their fusion with recycling endosomes. This hypothesis is consistent with recent report showing, by immunofluorescence microscopy, that insulin accelerates interendosomal Glut4 traffic in L6 myoblasts (37). At the same time, the amount of cellugyrin-negative vesicles in insulin-treated cells is decreased to a much higher extent indicating that virtually all IRVs are translocated to the plasma membrane in response to insulin stimulation. Thus, given that Glut4 and associated proteins recycle constantly in stimulated adipocytes (25, 38, 39), insulin may up-regulate several if not all steps of the Glut4-mediated pathway are related to traditional PI 3-kinase-mediated pathway are related to traditional PI 3-kinase-mediated signaling pathway for Glut4 translocation (38). Recently, a novel PI 3-kinase-independent signaling pathway for Glut4 translocation has been discovered (41). At present, it is not very clear how all these signaling pathways including the novel CAP-mediated pathway are related to traditional PI 3-kinase-mediated signaling. One attractive hypothesis is that different insulin signaling pathways work in concert but signal to different intracellular Glut4 compartments. For example, the novel Cbl/CAP-mediated pathway may trigger exocytosis of the preformed IRVs, whereas the traditional PI 3-kinase/Akt-mediated pathway may accelerate the transition of Glut4 from early/sorting endosomes to recycling endosomes or vice versa.

Yet another interesting observation is the following. Small cellugyrin-positive vesicles have not more than one molecule of either Glut4 or IRAP per vesicle. Larger cellugyrin-negative vesicles have a much higher specific content of these proteins (Fig. 4). At the same time, the total amount of Glut4 and IRAP found in the former compartment is the same or even greater than in the latter (Fig. 1). This suggests that, in the cell, there are several times more of the smaller, early endosome-derived cellugyrin-positive vesicles than the larger cellugyrin-negative IRVs.

Finally, our results may explain why insulin stimulates recycling of TfR and MPR in adipocytes as described previously in detail by several research groups (6–9). We suggest that, unlike Glut4 and IRAP, these receptors are not actively sorted into IRVs, but a fraction of both TfR and MPR can still penetrate into this insulin-responsive compartment (Figs. 1 and 3). Thus, in adipose cells, TfR and MPR may recycle through both Glut4-committed and non-committed, ubiquitous endosomal pathways (see also Refs. 25, 28). This may explain the fact that overall recycling of TfR and MPR in adipocytes demonstrate some insulin responsiveness, which is, however, less than the insulin responsiveness of Glut4 and IRAP that recycle exclusively via the regulated “specialized” pathway.

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