Cellugyrin Is a Marker for a Distinct Population of Intracellular Glut4-containing Vesicles*

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Tatyana A. Kupriyanova and Konstantin V. Kandror‡
From Boston University School of Medicine, Boston, Massachusetts 02118

Although Glut4 traffic is routinely described as translocation from an “intracellular storage pool” to the plasma membrane, it has been long realized that Glut4 travels through at least two functionally distinct intracellular membrane compartments on the way to and from the cell surface. Biochemical separation and systematic studies of the individual Glut4-containing compartments have been limited by the lack of appropriate reagents. We have prepared a monoclonal antibody against a novel component protein of Glut4 vesicles and have identified this protein as cellugyrin, a ubiquitously expressed homologue of a major synaptic vesicle protein, synaptogyrin. By means of sucrose gradient centrifugation, immunoadsorption, and confocal microscopy, we have shown that virtually all cellugyrin is colocalized with Glut4 in the same vesicles. However, unlike Glut4, cellugyrin is not re-distributed to the plasma membrane in response to insulin stimulation, and at least 40–50% of the total population of Glut4 vesicles do not contain this protein. We suggest that cellugyrin represents a specific marker of a functionally distinct population of Glut4 vesicles that permanently maintains its intracellular localization and is not recruited to the plasma membrane by insulin.

In fat, skeletal muscle, and heart, insulin causes massive translocation of Glut4-containing membrane vesicles to the plasma membrane (1–6). Several lines of evidence suggest that Glut4 travels through different intracellular membrane compartments during its recycling to and from the cell surface. First, Slot et al. (7, 8) showed by immunoelectron microscopy that intracellular Glut4 resides in different types of membrane structure. Second, the results of the mathematical analysis of Glut4 traffic are inconsistent with the existence of only one intracellular pool of Glut4, suggesting that there may be two or more distinct intracellular Glut4-containing compartments (9). Third, the analysis of targeting sequences in the Glut4 molecule lead to a three-pool model of Glut4 recycling (one plasma membrane pool and two intracellular pools) (10). Fourth, ablation of recycling endosomes with HRP3-conjugated transferrin resulted in only partial removal of Glut4 (11, 12), suggesting that Glut4 is present in recycling endosomes as well as in an unidentified separate compartment. Finally, biochemical separation of two (13) or even three (14) intracellular Glut4-containing compartments has recently been reported.

The nature of these multiple compartments is not completely understood. One of them, however, has been identified as early/sorting endosomes (7, 11, 12), and another may represent specialized post-endosomal insulin-responsive vesicles, or IRV (10–14). Mainly because of the lack of appropriate reagents, neither compartment has ever been purified and systematically studied, and therefore it is not clear what the biochemical difference between them may be.

To identify proteins that reside in different populations of Glut4 vesicles and may thus be used as specific protein markers for individual Glut4-containing compartments, we prepared monoclonal antibodies against several peripheral membrane proteins of Glut4 vesicles. We selected a hybridoma producing a monoclonal antibody against p28, a protein that has previously not been detected in Glut4 vesicles. Using this antibody, we immunopurified this protein from intracellular microsomes and identified it as cellugyrin by proteolytic digestion and mass spectrometry. This result was confirmed by expression of the cellugyrin cdNA (15) in COS cells. Cellugyrin represents a ubiquitous homologue of synaptogyrin (16), a major constituent of synaptic vesicles that is involved in regulation of exocytosis in PC12 cells (17). In rat adipocytes, at least 90% of cellugyrin is localized in Glut4-containing vesicles, whereas only 50–60% of Glut4 vesicles contain this protein. Unlike Glut4, cellugyrin is not redistributed to the plasma membrane in response to insulin stimulation. These data strongly indicate that intracellular Glut4-containing vesicles are not homogenous but represent a mixture of at least two individual populations: cellugyrin-negative vesicles, which are recruited to the cell surface in response to insulin stimulation; and cellugyrin-positive vesicles, which are not translocated by insulin. Interestingly, cellugyrin-positive vesicles are enriched with SCAMPs and VAMP2 and practically lack VAMP3/cellubrevin.

**EXPERIMENTAL PROCEDURES**

**Materials—**In the present study, we used monoclonal anti-Glut4 antibody 1F8 (18) for Western blotting and anti-Glut4 rabbit serum (19) (a kind gift of Dr. Giulia Baldini, Columbia University, New York) for immunofluorescent staining. Anti-IRAP rabbit serum was a kind gift of Dr. P. Pilch, Boston University School of Medicine, Boston, MA. Anti-cellubrevin rabbit serum was a kind gift of S. Olken and Dr. R. Corley, Boston University School of Medicine. DEAE-cellulose purified anti-IGF-II/IIa receptor polyclonal antibody was a kind gift of Dr. M. Czech, University of Massachusetts Medical School, Worcester, MA.

secretory carrier-associated membrane protein; VAMP, vesicle-associated membrane protein; IRAP, insulin-regulated aminopeptidase; v-SNARE, vesicle-soluble N-ethylmaleimide-sensitive factor-attachment protein receptor; LM, light microsomes; HM, heavy microsomes; PBS, phosphate-buffered saline.

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‡ To whom correspondence should be addressed: Dept. of Biochemistry, Boston University School of Medicine, K121 715 Albany St., Boston, MA 02118. Tel.: 617-638-5049; Fax: 617-638-5339; E-mail: kandror@biochem.bumc.bu.edu.

† The abbreviations used are: HRP, horseradish peroxidase; SCAMP, synaptosomal cytoplasmic protein.
Cellugyrin in Glut4 Vesicles

Rabbit anti-serum against soritin was prepared by Quality Controlled Biochemicals, Inc., Hopkinton, MA using the peptide ac-CP-GQQSKLYRSEDYGNFKPD-amide (amino acids 17–34) as antigen. Monoclonal antibody against SCAMPs was described earlier (20). The monoclonal anti-transferrin receptor antibody was from Zymed Laboratory, Inc., and monoclonal anti-VAMP2 antibody was from Synaptic Systems.

The rat cellugyrin cDNA in the pCMV5 expression vector was a kind gift of Dr. Thomas Sudhof, Southwestern Medical Center at Dallas, TX.

Preparation, Isotyping, and Purification of a Monoclonal Antibody against p28—Glut4-containing vesicles were immunosolubilated from rat adipocytes on acrylic immunobeads as described below and used for intracellular injection of BALB/c mice according to the protocol described by Thoidiis et al. (20) but without adjuvant. Mice were injected with the same amount of material (vesicles isolated from 0.5 mg of light microsomes (LM) on 0.2 ml of 1F8(4 heads) 4 times with 3-week intervals. The fusion of spleenocytes with SP2/0 mouse myeloma cells (2 spleen cells/1 myeloma cell) was performed 4 days after the final boost. The supernatants from resulting hybridomas were screened in a Mini-FRO-TEAN II multiscreen apparatus (Bio-Rad) by Western blotting using sucrose gradient-enriched (see below) and/or immunosolubilized Glut4-containing vesicles. A positive hybridoma selected for this study was cloned by limiting dilution. Hybridoma culture supernatant was isotype-d with the help of the Sigma ImmunoType kit (ISO-1) and the anti-p28 antibody was classified as IgG2a. This antibody was purified from the supernatant on immobilized protein A with the help of ImmunoPure (A) IgG Purification Kit (Pierce). In parallel, nonspecific mouse IgG were purified from mouse serum (Sigma). The purity of the isolated immunoglobulins was confirmed by SDS-electrophoresis and Coomasie staining.

Transfection of COS Cells—The rat cellugyrin cDNA was transfected into COS cells (4 μg of cDNA/100-mm dish) using Lipofectamine PLUS reagent (Life Technologies, Inc.) according to the manufacturer’s instructions. Dulbecco’s modified Eagle’s medium with 20% fetal bovine serum (5 ml) was added 3–5 h after transfection; 10% fetal bovine serum in Dulbecco’s modified Eagle’s medium was substituted the next day. Cells were harvested 48 h after transfection, lysed with 1 ml of extraction buffer (Tris-buffered saline with 1% Triton X-100, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 1 μg/ml pepstatin, 1 μg/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride) for 10–15 min on ice, and microfuged for 5 min. The supernatant was analyzed by Western blot.

Isolation of p28 from Rat Liver Microsomes—A microsomal membrane fraction was isolated from rat liver according to Fleischer and Kervina (21). The microsomal pellet (40 mg) was solubilized in PBS with protease inhibitors and 1% Triton X-100 for 4 h at 4 °C, centrifuged at 15,000 rpm for 30 min, and pre-cleared with nonspecific IgG coupled to acrylic beads. The resulting supernatant was incubated with 1 ml of anti-p28 immunobeads in a total volume of 10 ml overnight at 4 °C on a rotator. Immunobeads were then transferred to the column and washed with 15 ml of 10 mM phosphate buffer, pH 6.8. Elution was carried out with 100 mM glycine, pH 2.5; three 3-ml fractions were collected. Eluted proteins were precipitated with three volumes of cold ethanol, washed at −20 °C, pelleted at 15,000 rpm for 30 min, solubilized in 200 μl of Laemmli sample buffer (22), and separated by SDS-electrophoresis. The gel was stained with freshly prepared Coo-

FIG. 1. Anti-p28 antibody recognizes recombinant cellugyrin. The rat cellugyrin cDNA in the pCMV5 expression vector was transfected into COS cells as described under “Experimental Procedures,” and the total extract from transfected (+) and nontransfected (−) cells (25 and 50 μg, respectively) along with rat LM (100 μg) was analyzed by Western blotting 48 h later.

homogenized with a Potter-Elvehjem Teflon pestle, and subcellular fractions prepared as described previously (23).

Preparation of Tissue Extracts—Rat tissues were excised and immediately homogenized in a Polytron homogenizer at 13,500 rpm in HES buffer on ice. The homogenate was centrifuged for 10 min at 1500 × g and the pellet discarded. The resulting supernatant was analyzed by SDS-electrophoresis and Western blotting with anti-cellugyrin antibodies.

Fractionation of Intracellular Microsomes in Sucrose Velocity Gradients—Light and heavy microsomes (LM and HM) from rat adipocytes, suspended in PBS (150–200 μl) with protease inhibitors, were loaded on 4.6-ml 10–30% continuous sucrose gradients (in PBS) and centrifuged at 48,000 rpm in a Beckman SW-50.1 rotor for 55 min at 4 °C. Microsomes from the gradient were collected into 25–30 fractions starting from the bottom of the tubes. The protein concentration and refractive index were measured in aliquots. The position of Glut4- and cellugyrin-containing vesicles was determined by Western blotting of the gradient fractions. All high speed centrifugations were performed in a Sorval Discovery 90 ultracentrifuge.

Immunoadsorption of Glut4- and Cellugyrin-containing Vesicles—Protein A-purified IF8 antibody, anti-cellugyrin antibody, and nonspecific mouse IgG were each coupled to acrylic beads (Reacti-gel G0000, Pierce) at a concentration of 0.90, 2.0, and 0.91 μg/ml of antibody/ml of resin, respectively, 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. 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 three times with PBS, and the adsorbed material was subsequently eluted with 1% Triton X-100 in PBS and Laemmli sample buffer without reductants in order to avoid dissociation of the coupled antibodies.

Immunofluorescent Staining—Isolated rat adipocytes were fixed and stained according to Malide et al. (24). Briefly, cells were fixed with 4% paraformaldehyde in PBS, pH 7.4, for 20 min at room temperature, washed 3 times with PBS, permeabilized with 0.1% Triton X-100 in PBS for 15 min at room temperature, and blocked with blocking solution (3% normal donkey serum and 1% bovine serum albumin) for 1 h at room temperature. The cells were stained with anti-cellugyrin antibodies (10 μg/ml) and fluorescein isothiocyanate-conjugated donkey anti-mouse IgG (Jackson ImmunoResearch, 1:200) and then with polyclonal anti-Glut4 rabbit serum (1:1000) and Cy3-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch, 1:100). All dilutions of antibodies were done with the blocking solution. Each incubation with primary or secondary antibody lasted 1 h at room temperature. SlowFade-Light Antifade kit (Molecular Probes) was used for mounting cells on slides. Staining was examined using a confocal laser scanning microscope (Zeiss LSM 510).

GeL Electrophoresis and Immunoblotting—Proteins were separated in SDS-polyacrylamide gels according to Laemmli (22), but without reducing agents, and were transferred to Immobilon-P membrane (Millipore) in 25 mM Tris, 192 mM glycine. Following transfer, the membrane was blocked with 10% nonfat dry milk in PBS for 2 h at 37 °C. Proteins were visualized with specific antibodies, HRP-conjugated secondary antibodies (Sigma), and an enhanced chemiluminescent substrate kit (PerkinElmer Life Sciences). Autoradiograms were quantitated in a computing densitometer (Molecular Dynamics).

Protein Content—Protein content was determined with the BCA kit (Pierce) according to the manufacturer’s instructions.
RESULTS

Glut4 vesicles were immunoadsorbed from intracellular light microsomes, or LM (1 mg) with 0.2 ml of 1F8-beads as described previously (25–30). This material was injected intraperitoneally into each of four mice for 3 times every 3–4 weeks. Hybridoma-producing anti-p28 antibody was selected and cloned. This antibody was purified from the tissue culture supernatant on immobilized protein A, coupled to acrylic beads, and used for immunoisolation of p28 from rat liver microsomes as described under “Experimental Procedures.” The presence of cellugyrin in the isolated material was determined by Dr. W. Lane and associates by proteolytic digestion and mass spectrometry. To confirm this result, we expressed the rat cellugyrin cDNA (a kind gift of Dr. T. Südhof) in COS-7 cells, which have virtually no endogenous cellugyrin (15). Fig. 1 demonstrates that our anti-p28 monoclonal antibody recognizes the recombinant protein, which strongly suggests that p28 is indeed cellugyrin.

We also analyzed the expression of p28 in different rat tissues using our antibody. We found that this protein is present in every tissue studied with the exception of brain, which completely matches the tissue expression pattern of cellugyrin (15, 31) and confirms that p28 represents a ubiquitously expressed analogue of the brain-specific protein synaptogyrin (data not shown).

To study the intracellular localization of cellugyrin in rat adipocytes, we fractionated these cells according to Simpson et al. (23) and determined, by Western blotting, the cellugyrin content in different subcellular fractions (Fig. 2). In both basal and insulin-stimulated adipocytes, cellugyrin is readily detectable only in LM and HM fractions (Fig. 2). Longer exposures of the film allow detection of this protein in the plasma membrane, where the specific content of cellugyrin is much lower than in intracellular HM and LM (not shown), which may be explained by cross-contamination between the fractions. In these experiments, we did not detect any significant redistribution of cellugyrin between subcellular fractions in response

Fig. 2. Subcellular distribution of Glut4 and cellugyrin. Rat adipocytes treated (+) and not treated (−) with insulin were fractionated into subcellular fractions by differential centrifugation as described under “Experimental Procedures.” The figure demonstrates Western blot of each fraction (50 μg). Both proteins were visualized by consecutive staining of the same membrane. A representative result of five independent experiments is shown. M/N, mitochondria/nuclei; Cyt., cytosol.

Fig. 3. Sucrose block of endocytosis does not lead to the accumulation of cellugyrin in the plasma membrane. The figure demonstrates Western blot of membrane fractions (50 μg) isolated from insulin-treated and untreated adipocytes incubated in the absence or in the presence of 0.45 M sucrose for 30 min at 37 °C. All proteins were visualized by consecutive staining of the same membrane. A representative result of three independent experiments is shown. TfR, transferrin receptor.

Fig. 4. Cellugyrin co-localizes with Glut4 upon sucrose gradient centrifugation. LM (0.2 mg, lane 1; 100 μg, lane 2; 200 μg, lane 3) in 0.5 ml of PBS with protease inhibitors were immunoadsorbed with a fixed amount (50 μl) of 1F8-beads. The beads were washed 5 times with 1 ml of PBS and eluted with 150 μl of 1% Triton X-100 in PBS and then with 150 μl of Laemmli sample buffer. Eluate from nonspecific IgG-beads does not demonstrate any specific staining and is not shown. A representative result of four independent experiments is presented.

Fig. 5. Cellugyrin is localized in Glut4 vesicles. LM (50 μg, lane 1; 100 μg, lane 2; 200 μg, lane 3) and HM (1 mg) were isolated from insulin-treated (+) and untreated (−) adipocytes, resuspended in 180 μl of PBS with protease inhibitors, loaded on a 10–30% sucrose gradient, and centrifuged for 55 min at 48,000 rpm in an SW-50 Beckman rotor. Gradients were fractionated, starting from the bottom of the tube, into 27 (LM) and 23 (HM) fractions, and the positions of the individual proteins were determined by Western blot. Both proteins were visualized by consecutive staining of the same membrane. A representative result of four independent experiments is shown.
to insulin stimulation.

The apparent absence of cellugyrin in the plasma membrane may have two explanations. First, cellugyrin may be a truly intracellular protein that is not recruited to the plasma membrane even in the presence of insulin. Second, cellugyrin may rapidly recycle between the plasma membrane and its intracellular compartment but with an internalization rate so high that cellugyrin is not accumulated at the plasma membrane in any detectable quantities. To discriminate between these two possibilities, we blocked clathrin-mediated endocytosis in adipocytes by sucrose as described previously by Hansen et al. (32). As seen in Fig. 3, incubation of cells at 37°C for 30 min in 0.45 M sucrose leads to the accumulation of recycling proteins, such as the transferrin receptor and Glut4, in the plasma membrane. However, only traces of cellugyrin can be detected in this fraction under all experimental conditions. A similar result was obtained upon inhibition of clathrin-mediated endocytosis by potassium depletion (32) (results not shown); this suggests that cellugyrin is localized mainly in intracellular membranes both in the absence and presence of insulin.

To further explore the intracellular localization of cellugyrin, we carried out sucrose gradient centrifugation of LM and HM fractions (Fig. 4). We found that cellugyrin is present in rather homogenous vesicles, which are very well separated from the total microsomal protein (not shown) and demonstrate a high degree of overlap with Glut4-containing vesicles in both insulin-treated and nontreated cells.

The aim of the following experiments was to confirm co-localization of cellugyrin with Glut4 by immunoadsorption. As seen in Fig. 5, anti-Glut4 antibody 1F8 can efficiently immunoadsorb cellugyrin from the LM (and also HM, not shown) fraction. As expected, cellugyrin can be eluted from 1F8-beads with Triton X-100, whereas Glut4 is resistant to Triton elution and can be removed from the beads only with SDS-containing sample buffer. We estimated by densitometry of autoradiograms that at least 90% of the total p28 in LM is localized in Glut4 vesicles. For example, when 50 μg of LM were taken for 50 μl of 1F8-beads (Fig. 5, lane 1), we were able to immunoadsorb 91% of the total Glut4 and 85% of total cellugyrin. When
The protein composition of cellugyrin-containing vesicles. LM (100 μg) from insulin-treated and untreated rat adipocytes were immunoabsorbed with anti-cellugyrin immunobeads and with 1F8-beads (100 μl each). Beads were washed with PBS and eluted with 200 μl of 1% Triton X-100 in PBS and then with 200 μl of Laemmli sample buffer. Eluted proteins were separated in a 6.8% gel (for the detection of the IGFII/man 6-P receptor, the transferrin receptor (TfR), IRAP, and sortilin) and in a 10% gel (for the detection of Glut4, cellugyrin, SCAMPs, VAMP2, and VAMP3). The figure shows a combined Western blot of the two resulting membranes. All proteins except for VAMP2 (stained with alkaline phosphatase-conjugated secondary antibody) were visualized with HRP-conjugated secondary antibodies. Eluate from nonspecific IgG-beads does not demonstrate any specific staining and is not shown. A representative result of three independent experiments is presented.

we increased the amount of LM for the same volume of beads (lanes 2 and 3), their binding capacity was exceeded, and some Glut4 vesicles did not bind to the beads. Under all conditions, however, we immunoabsorbed identical fractions of Glut4 and cellugyrin. However, when we carried out immunoadsorption with anti-cellugyrin immunobeads, we were able to bring down only about 50–60% of Glut4-containing membranes (Fig. 6). We suggest, therefore, that cellugyrin is present only in a sub-population of Glut4-containing vesicles.

This result was confirmed by double-immunofluorescent staining of Glut4 and cellugyrin in rat adipocytes. With the help of confocal microscopy (Fig. 7), we show that a high degree of co-localization exists between cellugyrin and Glut4 in these cells. It is seen in Fig. 7, however, that although virtually all cellugyrin is co-localized with Glut4, a significant fraction of Glut4-containing membranes (about 40–50%) does not contain cellugyrin, which is consistent with the results of the immunoadsorption experiments. Interestingly, the middle section of Fig. 7 shows that Glut4-positive cellugyrin-negative vesicles are located closer to the cell surface than vesicles that contain both proteins. This observation may support indirectly the results of the subcellular fractionation (Figs. 2 and 3), which shows virtually no redistribution of cellugyrin to the plasma membrane in response to insulin.

We have found that all major recycling proteins previously found in Glut4 vesicles, such as IRAP, sortilin, receptors for transferrin, and IGFII/man 6-P are present in cellugyrin-containing vesicles (Fig. 8). Unexpectedly, it turned out that cellugyrin-positive vesicles are enriched in SCAMPs and VAMP2 but do not have (or have only traces of) VAMP3 (cellubrevin). In agreement with these data, VAMP3 is translocated to the cell surface in response to insulin to a greater extent than SCAMPs and VAMP2 (Fig. 9; see also Refs. 33 and 20). These results are consistent with the earlier observation that VAMP2 and VAMP3 are localized in different vesicular populations (33) and may or may not indicate that in rat adipocytes, VAMP3 rather than VAMP2 plays the role of the v-SNARE in the process of Glut4 vesicle fusion with the plasma membrane. Other studies performed in 3T3-L1 adipocytes (12, 34) and in L6 myoblasts (35) suggest, however, that VAMP2 is the v-SNARE in Glut4 vesicles. We believe that more experiments are required to determine the functions of VAMP isoforms in individual populations of Glut4 vesicles.

**DISCUSSION**

We have developed a monoclonal antibody against a previously unknown protein in Glut4 vesicles and have identified this protein as cellugyrin, a homologue of a major synaptic vesicle protein, synaptogyrin. Synaptogyrin, a four-transmembrane protein, is distantly related to synaptophysin (16) and represents one of the most abundant proteins in synaptic vesicles. Its biological functions are not exactly clear. In a recent report, exogenously expressed synaptogyrin was shown to potentially and specifically inhibit Ca<sup>2+</sup>-dependent exocytosis from PC12 cells (17). Knock-out experiments, however, have not revealed major biochemical changes in neurotransmitter release but have shown severely reduced short term and long term synaptic plasticity (36).

In fat cells (and also in skeletal and cardiomyocytes; not shown), virtually all cellugyrin is co-localized with Glut4. However, about 40–50% of Glut4 vesicles do not contain cellugyrin. Moreover, we have not detected any significant redistribution of cellugyrin to the plasma membrane in response to insulin stimulation; this suggests that cellugyrin may, in fact, be pres-
ent only in a distinct population of Glut4 vesicles with unique and specific properties. The possibility should be acknowledged that some Glut4 vesicles lack cellugyrin simply because its level of expression is low, so that not all Glut4 vesicles may have a copy of this protein. For the following reasons, however, we believe that cellugyrin-positive vesicles represent a distinct and independent compartment in adipose cells. First, their functional properties are different, because these vesicles are not translocated to the plasma membrane (Figs. 2 and 3). Second, cellugyrin-positive vesicles have unique and specific protein composition. We have shown that in addition to cellugyrin, these vesicles compartmentalize a significant fraction of Glut4-associated SCAMPS and VAMP2 and have no (or only traces of) VAMP3 (Fig. 8).

The majority of previously obtained results are consistent with the hypothesis that the total pool of intracellular Glut4 is distributed between early/sorting endosomes and putative post-endosomal vesicles that are recruited to the cell surface in response to insulin (see the Introduction). Based on our data, it seems logical to propose that cellugyrin may be localized specifically in the former compartment. Because Glut4 constantly recycles to and from the cell surface both in the absence and in the presence of insulin (37–39), its transition through the endosomal compartment should be accelerated by insulin. This, by the way, may be illustrated by Fig. 8, which shows that the amount of recycling proteins, such as the IGFII/man 6-P receptor, the transferrin receptor, IRAP, sortilin, and Glut4 itself, is significantly decreased in cellugyrin-positive vesicles after insulin administration. We believe, therefore, that the Glut4-containing endosomal compartment, although not translocatable directly to the plasma membrane, may still respond to insulin stimulation in as yet unknown fashion.

It was also suggested that intracellular Glut4 may be present not only in the endosomal compartments but in the trans-Golgi network as well (3). Others, however, have not detected trans-Golgi network markers in Glut4 vesicles (40, 41). The possibility still remains that cellugyrin-positive vesicles may represent a fraction of the trans-Golgi network; we will try to determine the cell biological nature of the individual Glut4-containing compartments in the near future.

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