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Characterization of Two Distinct Intracellular GLUT4 Membrane Populations in Muscle Fiber. Differential Protein Composition and Sensitivity to Insulin*

LIDIA SEVILLA†, EVA TOMÀS‡, PURIFICACIÓN MUÑOZ, ANNA GUMÀ, YVAN FISCHER, JULIA THOMAS, BONAVENTURA RUIZ-MONTASELL, XAVIER TESTAR, MANUEL PALACÍN, JOAN BLASI, AND ANTONIO ZORZANO

Departament de Bioquímica i Biologia Molecular (L.S., E.T., P.M., A.G., X.T., M.P., A.Z.), Facultat de Biologia, Universitat de Barcelona, Avinguda, Diagonal 645, 08028 Barcelona, Spain; Departament de Biologia Cellular i Anatomia Patològica (B.R.-M., J.B.), Universitat de Barcelona, Barcelona, Spain; and Institute of Physiology (Y.F., J.T.), Medical Faculty, Rheinisch-Westfälische Tecnische Hochschule Aachen, Pauwelsstrasse 30, D-52057 Aachen, Germany

ABSTRACT

A major objective for the understanding of muscle glucose disposal is the elucidation of the intracellular trafficking pathway of GLUT4 glucose carriers in the muscle fiber. In this report, we provide functional and biochemical characterization of two distinct intracellular GLUT4 vesicle pools obtained from rat skeletal muscle. The two pools showed a differential response to insulin; thus, one showed a marked decrease in GLUT4 levels but the other did not. They also showed a markedly different protein composition as detected by quantitative vesicle immunoisolation analysis. The GLUT4 pool showing no response to insulin contained SCAMP proteins and the vSNARE proteins VAMP2 and cellubrevin, whereas only VAMP2 was found in the insulin-recruitable GLUT4 pool. SDS-PAGE and further silver staining of the immunoprecipitates revealed discrete polypeptide bands associated to the insulin-sensitive pool, and all these polypeptide bands were found in the insulin-insensitive population. Furthermore, some polypeptide bands were exclusive to the insulin-insensitive population. The presence of cellubrevin and SCAMP proteins, endosomal markers, suggest that the insulin-insensitive GLUT4 membrane population belongs to an endosomal compartment. In addition, we favor the view that the insulin-sensitive GLUT4 membrane pool is segregated from the endosomal GLUT4 population and is undergoes exocytosis to the cell surface in response to insulin.

Intracellular GLUT4 membranes obtained from skeletal muscle contain cellubrevin, and VAMP2 and GLUT4-vesicles from cardiomyocytes also contain cellubrevin. This suggests that vSNARE proteins are key constituents of GLUT4 vesicles. The presence of the tSNARE protein SNAP25 in skeletal muscle membranes and SNAP25 and syntaxin 1A and syntaxin 1B in cardiomyocyte plasma membranes further suggest a role of the SNAREs in GLUT4 trafficking in muscle. (*Endocrinology* **138:** 3006–3015, 1997)

MUSCLE MAINTAINS glucose transport through the catalytic activity of two distinct glucose transporter isoforms, *i.e.* GLUT4 and GLUT1. The level of expression of GLUT4 and GLUT1 in muscles is developmentally regulated (1–3). In adult life, GLUT4 is the main glucose carrier expressed in skeletal muscle, accounting for nearly 90% of total glucose carriers (4). Under basal conditions, GLUT1 and GLUT4 show a different subcellular distribution in skeletal muscle. Thus, GLUT1 is found mainly in the sarcolemma of the muscle fiber, but not in transverse tubules (4, 5). In contrast, GLUT4 is mostly associated with intracellular membranous structures as detected by immunoelectron microscopy of human vastus lateralis (6), rat soleus (7–9), and rat extensor digitorum longus muscles (10). Intracellular GLUT4 is found in a perinuclear location close to Golgi and in the proximity of the sarcolemma or the transverse tubules in rat extensor digitorum longus muscle (10).

Currently available data indicate that insulin and exercise cause the translocation of GLUT4 glucose carriers. Immunocytochemical studies (6–9), subcellular fractionation (1, 11–16), and photolabeling assays (17, 18) indicate that GLUT4 translocates from an intracellular locus to the cell surface of the muscle fiber in response to insulin or exercise. Regarding the cell surface involved in insulin-induced GLUT4 recruitment, it has been shown that GLUT4 is recruited to selective domains of the sarcolemma (7–9, 15). Insulin also promotes the translocation of GLUT4 to the T-tubules of the muscle fiber as found by subcellular fractionation (15, 19), immunoisolation of T-tubule vesicles (15), immunoelectron microscopy (6), or 2-[^{3}H]-2-*N*-4(1-azi-2,2,2-tripluoraethyl)benzoyl-1, 3-bis (D-mannos 4-yloxy)-2-pro (ATB-[2-^{3}H]BMPA) photolabeling followed by autoradiography (20).

To delineate the intracellular trafficking pathway of GLUT4, two experimental strategies have been used in adipocytes. Firstly, the proteins that colocalize with GLUT4 in the same vesicles have been characterized. This

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Address all correspondence and requests for reprints to: Antonio Zorzano, Ph.D., Departament de Bioquímica i Biologia Molecular, Facultat de Biologia, Universitat de Barcelona, Avinguda, Diagonal 645, 08028 Barcelona, Spain. E-mail: azorzano@porthos.bio.ub.es.

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type of analysis has identified proteins such as phosphatidylinositol 4-kinase (21), SCAMPs (22, 23), gp160 (24–26), low molecular GTP-binding proteins including rab4 (27, 28), and the vSNARE proteins VAMP2 (29) and cellubrevin (30), which might be important in the mechanism of GLUT4 trafficking. Secondly, modeling analyses have shown that intracellular GLUT4 might involve two different pools in adipocytes. Thus, mathematical analysis of kinetic data of GLUT4 endocytosis and exocytosis obtained with the photoaffinity reagent ATB-BMPA (31, 32) predicts that GLUT4 localizes to at least two intracellular compartments in fat cells (33). Additionally, data of subcellular trafficking of both GLUT4 and GLUT1 and chimeric transporters in adipocytes (34) are best explained by a model postulating two intracellular pools (35). According to the predictions of this model, GLUT4 is internalized to an endosomal compartment and then sorted into an insulin-recruitable compartment; in contrast, GLUT1 is endocytosed into the endosomal compartment and recycles from this compartment to the cell surface (35). Further support to the idea that GLUT4 is present in separate intracellular compartments comes from very recent compartment ablation analysis; thus, ablation of the endosomal compartment in 3T3-L1 adipocytes reduces by 40% the amount of cellular GLUT4 (36). However, the information available in muscle is very scarce in spite of its obvious importance in the regulation of whole-body glucose homeostasis.

The SNARE hypothesis (37) postulates that fidelity in every fusion step between donor and acceptor membranes in the intracellular trafficking pathway is achieved by molecular recognition between membrane proteins associated with each compartment. Once this interaction is established, effective fusion is promoted by the action of SNAPs and NSF. Although it has recently been reported that such NSF-dependent mechanism does not underlie some specific membrane-sorting pathways (38), a large body of data supports its role in several membrane fusion events. Indeed, the proteins originally identified as the v- and t-SNAREs involved in synaptic vesicle exocytosis in neurons (*i.e.* VAMP, syntaxin 1, and SNAP-25) have been detected in other cell types with regulated exocytosis, such as endocrine pancreatic cells (39, 40). Furthermore, some isoforms of v- and t-SNAREs such as cellubrevin or syntaxin 2–5 are involved in constitutive fusion events (41, 42).

In the present study, we provide biochemical and functional characterization of intracellular GLUT4 membrane populations obtained from rat skeletal muscle. We obtained two distinct intracellular GLUT4 pools from rat skeletal muscle: one shows a marked decrease in GLUT4 levels after insulin administration, and the other does not. They show a different protein composition as detected by quantitative vesicle immunoisolation analysis followed by Western blot or SDS-PAGE and silver staining. Furthermore, we provide evidence for the expression of v-SNARE and t-SNARE proteins in muscle. v-SNARE proteins colocalize with intracellular GLUT4 and tSNARES are found in cell-surface and intracellular membranes. These results suggest the involvement of the SNAP/SNARE mechanisms in the trafficking of GLUT4 in muscle.

Materials and Methods

Materials

[^{125}I] protein A was purchased from ICN Iberica (Barcelona, Spain). An enhanced chemiluminescence system was obtained from Amersham International plc (Buckinghamshire, UK). Immobilon PVDF was obtained from Millipore. Gamma-globulin, wheat germ agglutinin, goat antimouse IgG- and goat antimouse IgM-coupled to agarose and most commonly used chemicals were from Sigma Chemical Co. (St. Louis, MO). All chemicals for media used for cardiomyocyte isolation and subcellular fractionation were from Merck; aprotinin, pepstatin, and leupeptin were from ICN; BSA, fraction V, and fatty-acid free were purchased from Boehringer; purified porcine insulin was a kind gift from Eli Lilly (Indianapolis, IN). All chemicals were of the highest purity grade available. All electrophoresis reagents and mol wt markers were obtained from Bio-Rad Laboratories, S.A. (Madrid, Spain).

Antibodies

Both monoclonal (1F8) and polyclonal (OSCRX) antibodies specific for GLUT4 were used in these studies. Monoclonal antibody 1F8 (43) was used for immunoisolation assays and was kindly given by Dr. Paul F. Pilch (Department of Biochemistry, Boston University Medical School, Boston, MA). Anti-GLUT4 (OSCRX) from rabbit was produced after immunization with a peptide corresponding to the final 15 amino acids of the carboxyl terminus (44). Monoclonal antibody 3F8 was used to immunodetect GTV3/SCAMP proteins (22) and was also a kind gift of Dr. Paul F. Pilch. A rabbit polyclonal antibody against rat β_1-integrin was kindly given by Dr. Carles Enrich (University of Barcelona, Barcelona, Spain) (45). A rabbit polyclonal antibody against the α_2 component of Ca^{2+} channels (dihydropyridine receptors) (46) was obtained from Dr. Michel Lazdunski (Centre de Biochimie, Centre National de la Recherche Scientifique, Sophia Antipolis, France). Polyclonal antibody 18B11, against TGN 38, was kindly given by Dr. Ignacio Sandoval (Centro de Biología Molecular, Madrid, Spain). Monoclonal antibody NCL-DYS 1 against the mid rod of dystrophin was purchased from Novocastra, UK. Monoclonal antibody TT-2, which recognizes protein tt28 specific for T-tubules (47), was also used in these studies. A polyclonal antibody against the C-terminus of the rat insulin receptor (residues 1341–1357) was kindly given by Dr. Willy Stalmans (Katholieke Universiteit Leuven, Belgium) (48). VAMP-2 polyclonal antibody was a generous gift from Dr. R. Jahn (New Haven, CT). Polyclonal antibodies against cellubrevin were kindly given by P. De Camilli (New Haven, CT) (41). Syntaxin 1 monoclonal antibody (HPC-1) was generously given by C. Barnstable (New Haven). This antibody recognizes the two highly related isoforms syntaxin 1A and 1B. (49). Antiserum against SNAP-25 was raised in rabbits, as described elsewhere (50).

Animals and tissue sampling

Male Wistar rats weighing between 250 and 300 g from our own colony were fed with Purina Laboratory chow *ad libitum* and housed in animal quarters maintained at 22 C with a 12-h light, 12-h dark cycle. After an overnight fast, rats were anesthetized with sodium pentobarbital and some were injected with insulin (iv, 10 U/kg body weight) and D-glucose (ip, 1 g/kg body weight) 30 min before tissue removal. White portions of gastrocnemius and quadricep muscles were then rapidly excised and immediately processed.

Subcellular fractionation of rat skeletal muscle membranes

Several cell surface and intracellular membrane fractions were isolated as reported (5, 15, 51). Approximately 12 g of rat skeletal muscle was excised, weighed, minced, and initially homogenized with Polytron (Kinematica Gmbh, Switzerland) at low speed (setting 4, 2 × 5 sec) in buffer A (20 mM Tris-HCl, 0.25 M sucrose, 1 mM EDTA, 1 μM pepstatin, 1 μM leupeptin, pH 7.4; 1 g/4 ml). The homogenate was centrifuged for 20 min at 12,000 × *g* [9,000 rpm in SA-600 Sorvall (Wilmington, DE) rotor]. The supernatant was collected and kept on ice. The pellet was resuspended in buffer A and centrifuged again for 20 min at 12,000 × *g*. The two supernatants were pooled and were referred to as F1 fraction. The pellet was resuspended in buffer A and subjected to high-speed homogenization (Polytron at setting 6, 2 ×30 sec). The homogenate was

centrifuged for 20 min at 12,000 × *g*, and the supernatant was collected and referred to as F2. F1 and F2 fractions were incubated with 0.6 M KCl for 1 h at 4 C and then pelleted for 1 h at 150,000 × *g* in a T-647.5 Sorvall rotor. The pellets from KCl-washed F1 and F2 fractions were then subjected to calcium-loading to increase the density of sarcoplasmic reticulum vesicles (52). To this end, pellets were resuspended in buffer B (50 mM potassium phosphate, 5 mM $MgCl_2$, 150 mM KCl, pH 7.5) at a protein concentration of 2 mg/ml. Calcium loading was initiated by addition of 0.3 mM $CaCl_2$ and 2 mM ATP. After incubation for 20 min at room temperature, F1 and F2 fractions were kept on ice and centrifuged for 60 min at 150,000 × *g*. F1 and F2 pellets were resuspended in buffer C (20 mM Tris-HCl, 50 mM sodium pyrophosphate, 0.3 M KCl, 0.25 M sucrose, pH 7.2) and layered on top of a discontinuous density gradient consisting of 3 ml 35%, 2 ml 29%, 2 ml 26%, and 2 ml 23% (wt/vol) sucrose. After centrifugation for 12 h at 77,000 × *g* (25,000 rpm in a TH-641 Sorvall rotor), four protein fractions were separated from F1 and F2 fractions: fraction 23 on top of the 23% layer; fraction 26 from the interphase 23%–26%; fraction 29 from the interphase 26%–29%; fraction 35 from the interphase 29%–35%. In some experiments, the pellet resulting from this centrifugation was also collected (pellet-F1 and pellet-F2) (see Fig. 1). All the fractions were collected, diluted with 20 mM Tris-HCl, pH 7.4, and centrifuged for 60 min at 150,000 × *g*. Pellets were resuspended in 30 mM HEPES, 0.25 M sucrose, pH 7.4. Proteins were determined by the method of Bradford (53) using γ-globulin as a standard.

Isolation of cardiomyocytes and preparation of membrane fractions

Cardiomyocytes from females Sprague-Dawley rats (180–220 days old, fed *ad libitum*) were obtained as previously described (54). Isolated cardiomyocytes were incubated in medium containing 6 mM KCl, 1 mM Na_2HPO_4, 0.2 mM NaH_2PO_4, 1.4 mM $MgSO_4$, 128 mM NaCl, 10 mM HEPES, 1 mM $CaCl_2$ and 2% BSA, fatty acid-free, pH 7.4, in the absence (control) or in the presence of insulin (10 nM) at 37 C for 30 min. The cells were washed once with TES-buffer (20 mM Tris, 1 mM EDTA, 250 mM sucrose, pH 7.4) and then immediately frozen in liquid nitrogen in a ratio of 10^7 cells/2.7 ml TES.

All of the following steps were performed at 4 C as reported (55). For membrane fractionation, cells were rapidly thawed (for each condition 2×10^7 cells) and homogenized in a Potter-Elvenjhem (Braun-Melsungen; 30 ml; clearance 0.2 mm) in 11 ml TES-buffer at 180 rpm with 10 up-and-down strokes over 60 sec. These homogenates were centrifuged for 15 min at 17,000 × *g*. The pellet of this centrifugation (P1) was washed once with TES (12,000 rpm, 20 min), before it was resuspended in 1.5 ml TES, layered on a sucrose cushion [38% (wt/vol) sucrose, 20 mM Tris, 1 mM EDTA, pH 7.4] and ultracentrifuged for 65 min at 65,000 × *g*. The white interface was taken off, diluted with approximately 8 ml TES, and pelleted at 48,000 × *g* within 30 min, washed with 1 ml TES, and pelleted again in an Eppendorf tube at 48,000 × *g* for 30 min. This fraction (referred to as PM in the text) was enriched with the plasma membrane marker enzyme ouabain-sensitive p-nitrophenylphosphatase by a factor of 13.5, whereas the specific activity of the sarcoplasmic reticulum marker EGTA-sensitive Ca^{++}-ATPase was decreased by a factor of 3.6 when compared with crude cell homogenates.

To obtain a fraction containing the intracellular glucose transporter pool, the supernatant (S1) of the first centrifugation was centrifuged for 30 min at 48,000 × *g*, resulting in the separation of a high-density microsome fraction (which appears in the pellet of this centrifugation and is contaminated with plasma membranes) and a low-density membrane fraction in the supernatant (S2). S2 was finally ultracentrifuged for 65 min at 250,000 × *g*. In the resulting pellet (designated LDM in the text), no p-nitrophenylphosphatase or Ca^{++}-ATPase activity was detectable, indicating that this fraction was devoid of plasma membrane and of sarcoplasmic reticulum elements.

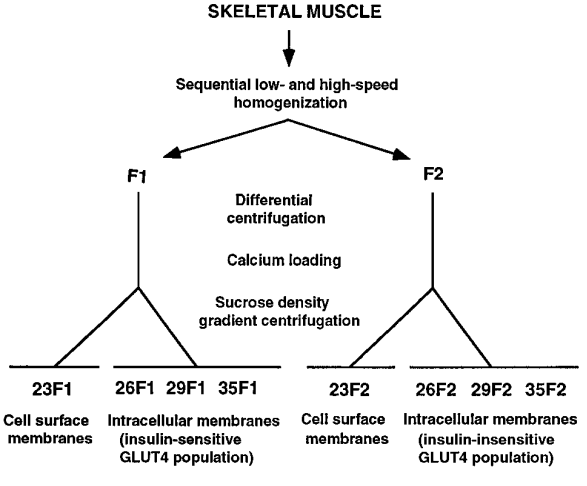

FIG. 1. Flow chart of procedure to isolate different membrane fractions from rat skeletal muscle.

Protocols of vesicle immunoisolation

Protein A-purified 1F8 antibody was coupled to acrylamide beads (Reacti-gel GF 2000, Pierce) at a concentration of 1 mg antibody per ml of resin according to manufacturer's instructions. Before use, the beads were saturated with 1% BSA in PBS (134 mM NaCl, 2.6 mM KCl, 6.4 mM Na_2HPO_4, 1.46 mM KH2PO4, pH 7.4) for at least 30 min at room temperature and then washed in PBS. Intracellular membranes were incubated with beads overnight at 4 C (50 μg of membranes and 20 μl of beads). The beads were spun down, the supernatant was taken for later analysis, and the beads were washed five times in PBS. The adsorbed material was eluted with electrophoresis sample buffer (0.1 M Tris-HCl, 20% glycerol and 2% SDS, pH 6.8), incubated for 5 min at 95 C, cooled, and microcentrifuged. The supernatant fraction from the vesicle immunoadsorption assay and the immunoadsorbed extract were subjected to SDS-PAGE.

Electrophoresis and immunoblot analysis

SDS-PAGE was performed on membrane protein following Laemmli (56). Tris-Urea/SDS-PAGE (18% acrylamide, 6 M urea, 750 mM Tris-HCl, pH 8.85, 50 mM NaCl, 0.1% SDS) was performed as previously described (57). High resolutive Tris-Urea/SDS-PAGE enables effective separation of syntaxin1A and syntaxin 1B isoforms, which otherwise appear as a single band of 35 kDa in standard 10–12.5% SDS/PAGE assay. With the former technique syntaxin 1A and syntaxin 1B appear as separate bands of 38 kDa and 46 kDa, respectively.

Proteins were transferred to Immobilon (Millipore Corp., Bedford, MA) as previously reported (58) in buffer consisting of 20% methanol, 200 mM glycine, 25 mM Tris, pH 8.3. Following transfer, the filters were blocked with 5% nonfat dry milk, 0.02% sodium azide in PBS for 1 h at 37 C and were incubated with antibodies in 1% nonfat dry milk, 0.02% sodium azide in PBS. Transfer was confirmed by Coomassie blue staining of the gel after the electroblot. Detection of the immune complex with the rabbit polyclonal antibodies was accomplished using ^{125}I-protein A for 4 h at room temperature. Detection of the immune complex with monoclonal antibodies was performed using sheep antimouse ^{125}I-antibody. Antibody 3F8 was detected using horseradish peroxidase linked to goat anti-IgM mouse secondary antibody and visualized using an enhanced chemiluminescence system. The autoradiograms were quantified using scanning densitometry. Immunoblots were performed under conditions in which autoradiographic detection was in the linear response range.

Results

Isolation of two distinct intracellular GLUT4 pools from rat skeletal muscle showing different insulin responsiveness

The distribution of GLUT4 in membrane fractions obtained from skeletal muscle from control or insulin-treated rats was studied. To this end, we used a previously published protocol of membrane fractionation (5, 15) that involves the preparation of two crude membrane fractions by sequential homogenization at low speed (F1) and high speed (F2) start-

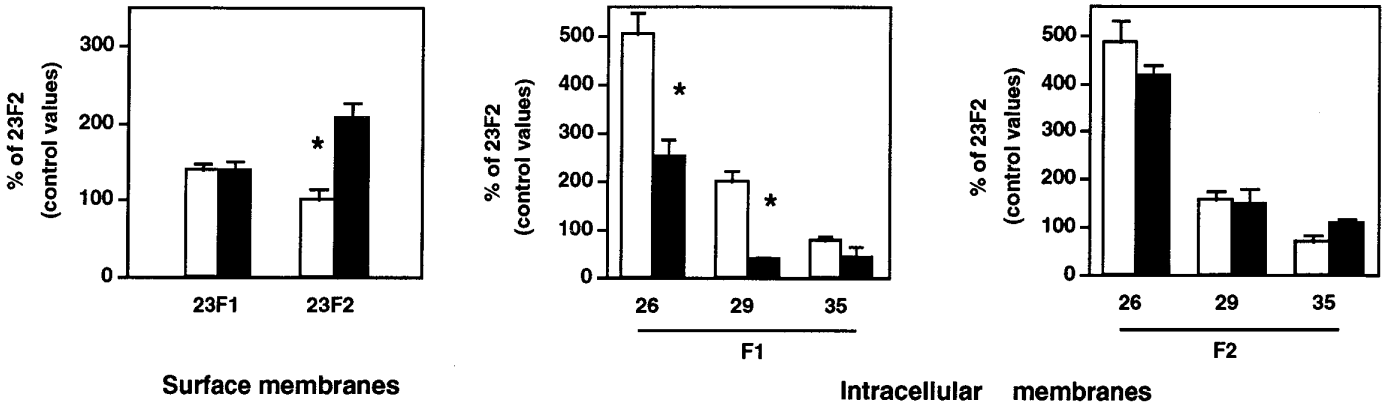

FIG. 2. Insulin recruits GLUT4 from intracellular membranes to the cell surface in skeletal muscle. The abundance of GLUT4 was assayed in 1) surface membrane fraction 23F1 enriched in sarcolemmal markers and in membrane fraction 23F2 enriched in sarcolemmal markers and in T-tubule markers; and 2) in intracellular membranes 26F1, 29F1, 35F1, 26F2, 29F2, and 35F2 from control and insulin-stimulated muscles. The distribution of GLUT4 was determined by immunoblot analysis by using a polyclonal antibody against the C-terminus of GLUT4. Equal amounts of membrane proteins (1 μg) from the different fractions were laid on gels. The results (mean + SEM) of four to seven experiments were expressed as a percentage of levels detected in fraction 23F2 (control group). *, Significant difference between control (*open bars*) and insulin-treated groups (*black bars*), at $P < 0.05$.

ing from a single preparation of muscle tissue (Fig. 1). Upon centrifugation in sucrose gradients, membrane collected from the top of the 23% sucrose layer (23F1 and 23F2) represents crude cell-surface fraction. Fraction 23F1 is enriched in sarcolemmal markers, and fraction 23F2 in enriched in both sarcolemmal and T-tubule markers (5, 15). Membrane fractions obtained from the top of denser sucrose layers (26F1, 29F1, 35F1 or 26F2, 29F2, 35F2) are enriched in intracellular markers (5, 15). Administration of a supramaximal dose of insulin (30 min after 10 U insulin/kg body weight, iv) increased GLUT4 in membrane fraction 23F2, which is enriched in sarcolemmal and T-tubule membranes (Fig. 2). This pattern was specific to the glucose transporter, and no effect of insulin was detected in the distribution of the surface markers β_1-integrin, tt28, dihydropyridine receptors or dystrophin (data not shown). Concomitantly, insulin treatment caused a marked decrease in the content of GLUT4 in intracellular vesicles derived from fractions 26F1, 29F1, and 35F1 (levels in 26F1, 29F1, and 35F1 fractions in the insulin-treated group were 40%, 19%, and 67% of values found in the control group) (Fig. 2). No effect of insulin was detected in the GLUT4 content of intracellular fractions 26F2, 29F2, or 35F2 (Fig. 2). The total amount of GLUT4 recovered from all fractions was not different in control and insulin-treated muscles (data not shown). In addition, approximately 70% of all intracellular GLUT4 was found in F2 membrane fractions.

These results suggest two distinct GLUT4 membrane populations: one that responds to insulin by decreasing GLUT4 (26F1, 29F1, and 35F1) and the other that is unresponsive to insulin (26F2, 29F2, and 35F2). To provide further evidence for a differential insulin responsiveness, GLUT4 vesicles were immunoisolated from 26F1 and 26F2 membrane fractions obtained from control and insulin-stimulated muscles (Fig. 3). Vesicle immunoisolation analysis was performed using antibody 1F8 (against GLUT4) coupled to acrylic beads. Antibody 1F8 immunoadsorbed nearly 90% and 89% of total GLUT4 from the fractions 26F1 and 26F2, respectively (data not shown). The level of nonspecific adsorption of GLUT4 was consistently greater in the 26F2 fraction than in

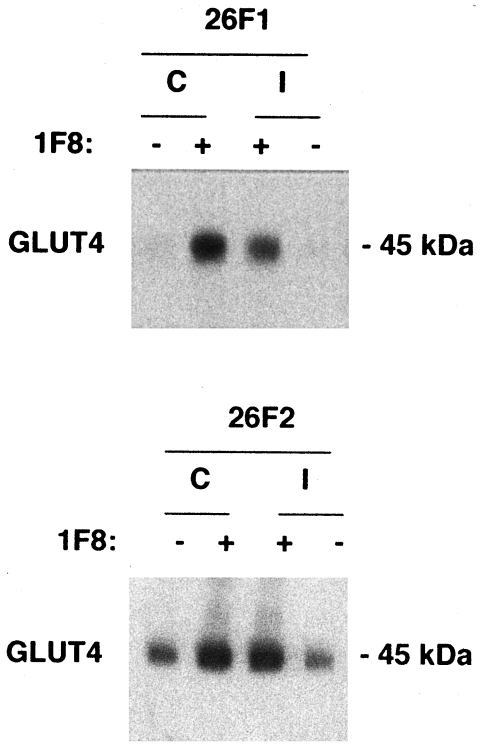

FIG. 3. Immunoadsorption of GLUT4 vesicles: insulin depletes GLUT4 in selective intracellular membrane populations. Membrane vesicles 26F1 (insulin-sensitive GLUT4 pool) and membrane vesicles 26F2 (insulin-insensitive GLUT4 pool) obtained from nonstimulated or insulin-stimulated skeletal muscles were incubated with (+) or without (−) antibody 1F8. After the incubation, the adsorbed fractions were electrophoresed and immunoblotted to determine the abundance of GLUT4. Autoradiographs were subjected to scanning densitometry. Representative autoradiograms, obtained after various times of exposure are shown.

the 26F1 membrane fraction (Fig. 3), and there is no obvious explanation for this. Furthermore, the amount of GLUT4 immunoisolated from fractions 26F1 or 29F1 was markedly decreased after insulin administration (Fig. 3 and data not

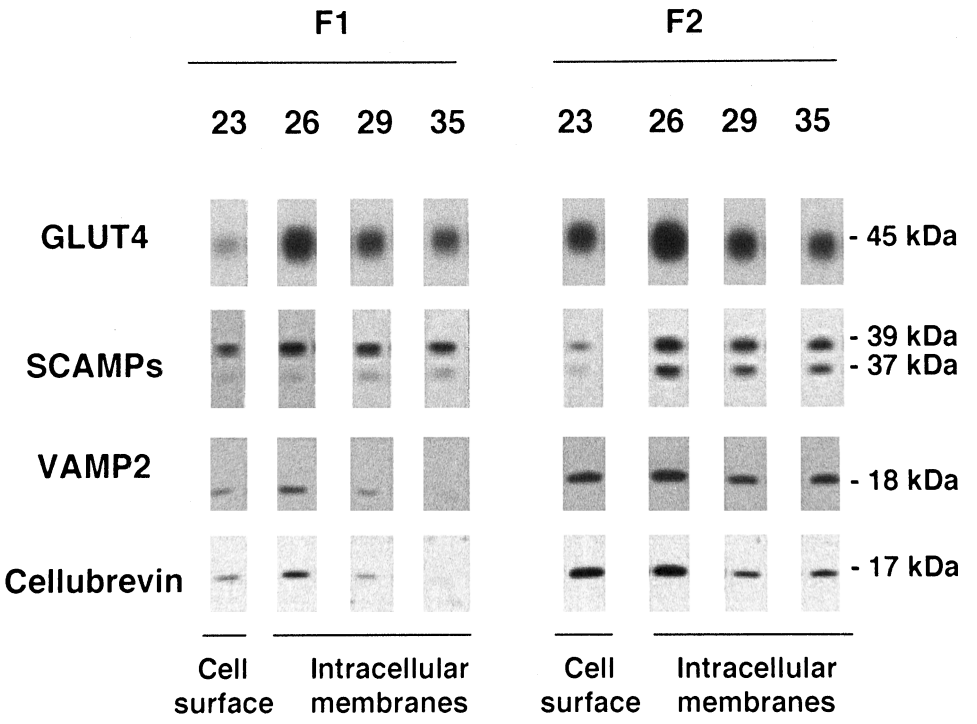

FIG. 4. Distribution of GLUT4, SCAMPs, cellubrevin, and VAMP2 in membranes from skeletal muscle. The abundance of GLUT4, SCAMPs, cellubrevin, and VAMP2 was assayed in surface membrane fractions 23F1 and 23F2 and in intracellular membranes 26F1, 29F1, 35F1, 26F2, 29F2, and 35F2 from rat skeletal muscle. The distribution of GLUT4, SCAMPs, cellubrevin, and VAMP2 was determined by immunoblot analysis by using specific antibodies (see *Materials and Methods*). Equal amounts of membrane proteins (4 μg) from the different fractions were laid on gels. Representative autoradiograms from four to seven experiments are shown.

shown). In contrast, no change in the amount of GLUT4 immunoadsorbed was found when comparing fractions 26F2 or 29F2 obtained from basal and insulin-treated groups (Fig. 3 and data not shown).

Thus, subcellular membrane fractionation confirmed the detection of two intracellular GLUT4 pools, only one of which responded to insulin. The two intracellular GLUT4 pools show a different protein composition. In subsequent experiments, we characterized the two intracellular GLUT4 pools obtained from rat skeletal muscle, *i.e.* the insulin-sensitive and the insulin-sensitive pools. This study was limited to fractions 26F1, 29F1, 26F2, and 29F2 because these membrane fractions were more highly enriched in GLUT4 than 35F1 or 35F2 (Fig. 2). In initial experiments, we determined the presence of SCAMP proteins, cellubrevin and VAMP2, which are reported to colocalize with GLUT4 vesicles in rat adipocytes (22, 23, 29, 30), in intracellular membranes obtained from rat skeletal muscle. These proteins were present in substantial amounts in muscle membranes and the pattern of distribution was similar to that detected for GLUT4 (Fig. 4). Thus, the abundance of these proteins was greater in intracellular membranes than in cell surface membranes (Fig. 4). Intracellular muscle membranes also contained insulin receptors and TGN 38 (data not shown).

Next, we determined whether SCAMPs, cellubrevin, or VAMP2 colocalize with GLUT4 in intracellular membranes. To this end, quantitative vesicle immunoisolation analysis using antibody 1F8 coupled to acrylic beads was performed in insulin-sensitive (*i.e.* fraction 26F1) or insulin-insensitive (*i.e.* 26F2) intracellular membrane fractions. As previously mentioned, this method of vesicle immunoisolation adsorbed nearly 90% of total GLUT4 from both fractions (Fig. 5). Under these conditions, SCAMP proteins were substantially immunoadsorbed only in fraction 26F2 (insulin-insensitive pool) (near 50% of total SCAMPs present in fraction 26F2 was specifically immunoadsorbed) (Fig. 5). Similar results were obtained when the colocalization between GLUT4 and cellubrevin was explored. Thus, only immunoisolated GLUT4-vesicles obtained from the insulin-insensitive pool contained substantial amounts of cellubrevin (near 20% of total cellubrevin present in the membrane fraction colocalized in GLUT4 vesicles) (Fig. 6). In contrast, VAMP2 protein was detected both in GLUT4 vesicles isolated from both fractions (near 30% of total VAMP2 present in the membrane

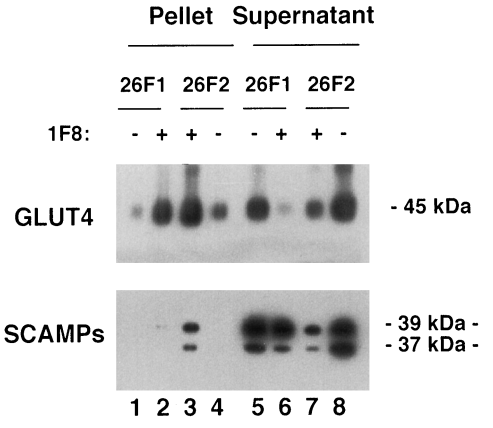

FIG. 5. Colocalization of GLUT4 and SCAMPs in the intracellular insulin-insensitive pool. Membrane vesicles 26F1 (insulin-sensitive GLUT4 membranes) and membrane vesicles 26F2 (insulin-insensitive GLUT4 membranes) obtained from nonstimulated skeletal muscle were incubated with (+) or without (−) antibody 1F8. After the incubation, the adsorbed (P) and nonadsorbed (S) fractions were electrophoresed and immunoblotted to determine the abundance of SCAMPs and GLUT4. Autoradiographs were subjected to scanning densitometry. Representative autoradiograms, obtained after various times of exposure are shown.

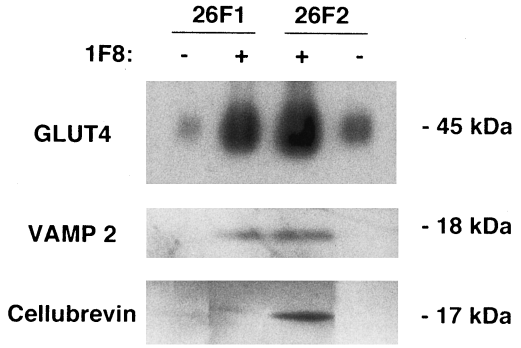

FIG. 6. Distinct colocalization pattern of cellubrevin and VAMP2 in intracellular insulin-sensitive and insulin-insensitive GLUT4 pools. Membrane vesicles 26F1 (insulin-sensitive GLUT4 membranes) and membrane vesicles 26F2 (insulin-insensitive GLUT4 membranes) obtained from nonstimulated skeletal muscle were incubated with (+) or without (−) antibody 1F8. After the incubation, the adsorbed fractions were electrophoresed and immunoblotted to determine the abundance of cellubrevin, VAMP2, and GLUT4. Autoradiographs were subjected to scanning densitometry. Representative autoradiograms, obtained after various times of exposure are shown.

fractions) (Fig. 6). No insulin receptors or TGN38 proteins were detected in GLUT4-vesicles derived from both pools (data not shown).

SDS-PAGE of the immunoprecipitates followed by silver stain was performed from insulin-sensitive (fractions 26F1 or 29F1) and insulin-insensitive membranes (fractions 26F2 or 29F2). A discrete number of bands was detected in the immunoprecipitates from insulin-sensitive fractions 29F1 (Fig. 7) or from 26F1 (data not shown), the most intense bands showing apparent mobility of near 220, 210, 158, 79, and 50 kDa. In some experiments, we also found a polypeptide band of 110 kDa in the insulin-sensitive GLUT4 pool (data not shown). The bands showing apparent mobility of 220, 210, 158, 79, and 50 kDa were also detected, although with greater abundance, in the GLUT4 vesicles obtained from insulin-insensitive fractions 29F2 (Fig. 7) or 26F2 (data not shown). Furthermore, some additional bands were detected in insulin-insensitive GLUT4 vesicles (derived from fraction 29F2) that were not found in the insulin-sensitive GLUT4 population (derived from fraction 29F1) (Fig. 7). The polypeptide bands specific of insulin-insensitive GLUT4-vesicles showed apparent mol wt of 63, 60, 32, 28, and 25 kDa (Fig. 7). In summary, these results show a differential protein composition of the insulin-sensitive and the insulin-insensitive intracellular GLUT4 populations.

Skeletal muscle and cardiomyocyte membranes contain vSNARE and tSNARE proteins

Based on the expression of cellubrevin and VAMP2 in skeletal muscle membranes, and especially the pattern of colocalization in intracellular GLUT4 vesicles, we asked next whether cellubrevin and VAMP2 proteins might represent general key constituents for GLUT4 trafficking in muscle cells. To this end, we studied the expression of these proteins in freshly isolated rat cardiomyocytes (Fig. 8). Cellubrevin but not VAMP2 was detected in cardiomyocyte membranes by immunoblotting (Fig. 8 and data not shown). Cellubrevin was mainly found in intracellular membranes, and insulin

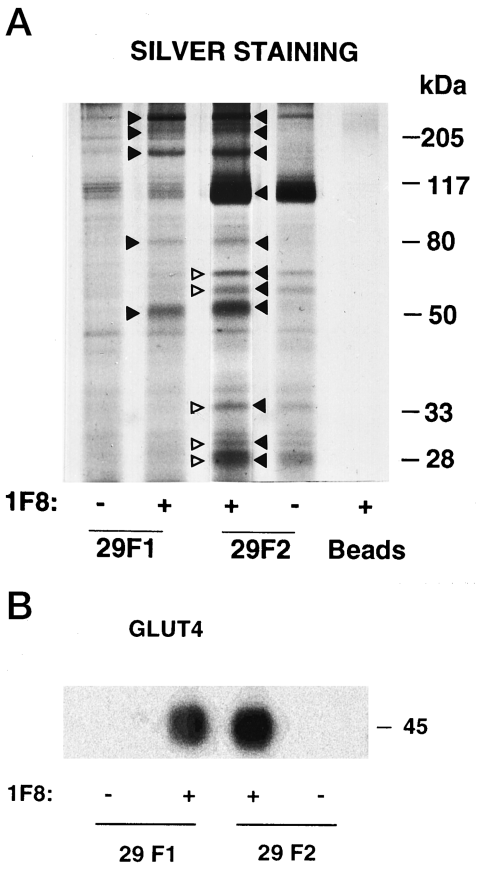

FIG. 7. Distinct pattern of polypeptide bands in intracellular insulin-sensitive and insulin-insensitive GLUT4 pools. Membrane vesicles 29F1 (insulin-sensitive GLUT4 pool) and membrane vesicles 29F2 (insulin-insensitive GLUT4 pool) obtained from nonstimulated skeletal muscle were incubated with (+) or without (−) antibody 1F8. After the incubation, the adsorbed fractions were electrophoresed and subjected to silver staining. Autoradiographs were subjected to scanning densitometry. Representative gels are shown. *Black arrows* indicate polypeptide bands present in GLUT4 vesicles; *open arrows* indicate the polypeptide bands that are specific of GLUT4 vesicles from fraction 29F2.

did not cause any modification in subcellular distribution (Fig. 8) in contrast to GLUT4 which was translocated from the intracellular membranes to the cell surface (Fig. 8). Next, we investigated whether GLUT4 and cellubrevin colocalized intracellularly by vesicle immunoisolation analysis. Results clearly indicated that under conditions in which near 65% of total GLUT4 was specifically immunoadsorbed (Fig. 8), about 25% of the total cellubrevin was specifically detected in the immunoprecipitates (Fig. 8). These results indicate the presence of the vSNARE protein cellubrevin, but not VAMP2, in intracellular GLUT4 vesicles obtained from cardiomyocytes and suggest differences in vSNARE composition of GLUT4 vesicles derived from skeletal and cardiac tissues.

In view of the presence of the vSNARE proteins, cellubrevin and VAMP2, in skeletal muscle and cellubrevin in cardiomyocytes, we searched for the presence of tSNARE proteins, syntaxin 1 and SNAP 25. SNAP 25 protein but not syntaxin 1 was detected in membranes obtained from rat skeletal muscle (Fig. 9). Furthermore, SNAP 25 was found

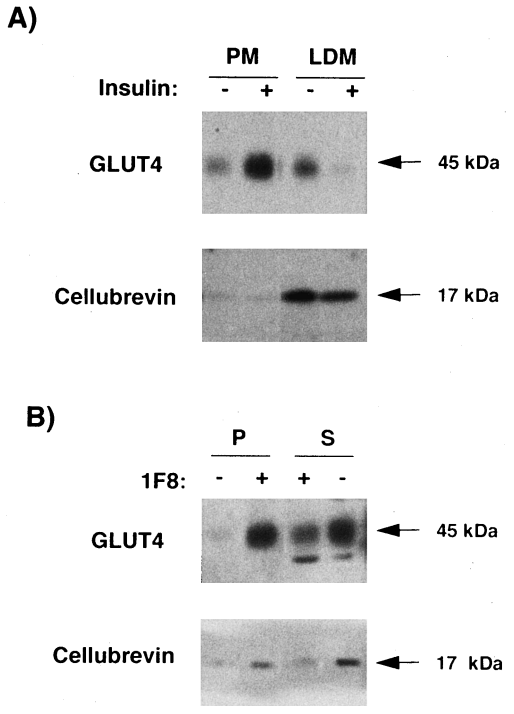

FIG. 8. Cellubrevin is expressed in isolated rat cardiomyocytes and colocalizes with intracellular GLUT4. The abundance of GLUT4 and cellubrevin was assayed in plasma membranes (PM) and intracellular membrane fractions (LDM) from unstimulated and insulin-treated cardiomyocytes (A). Equal amounts of membrane proteins (20 μg) from the different fractions were laid on gels. LDM membranes obtained from nonstimulated cardiac myocytes were immunoadsorbed with 1F8-acrylic beads (+) or with beads linked to nonspecific antibodies (−). After the incubation, the adsorbed (P) and nonadsorbed (S) fractions were electrophoresed and immunoblotted to determine the abundance of GLUT4 and cellubrevin (B). The amount of cellubrevin and GLUT4 was determined by immunoblot analysis by using specific antibodies. Representative autoradiograms, obtained after various times of exposure, are shown.

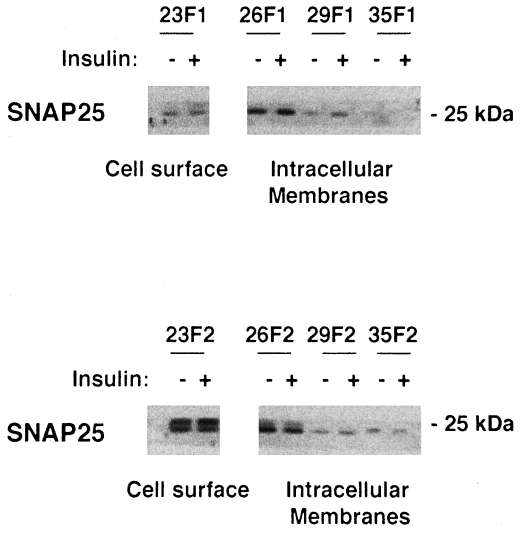

FIG. 9. Distribution of SNAP25 in skeletal muscle membranes. The abundance of SNAP25 was assayed in surface membrane fractions 23F1 and 23F2 and in intracellular membranes 26F1, 29F1, 35F1, 26F2, 29F2, and 35F2 from rat skeletal muscle. The distribution of SNAP25 was determined by immunoblot analysis by using specific antibodies (see *Materials and Methods*). Equal amounts of membrane proteins (4 μg) from the different fractions were laid on gels. Representative autoradiograms from four to seven experiments are shown.

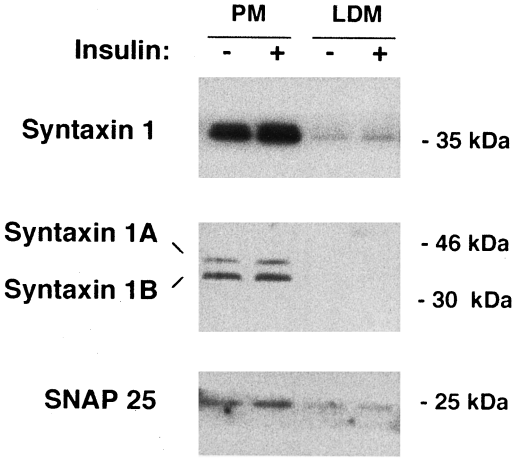

FIG. 10. Distribution of syntaxin 1A and 1B and SNAP25 in membranes from isolated rat cardiomyocytes. The abundance of syntaxin 1A and 1B and SNAP25 was assayed in plasma membranes (PM) and intracellular membrane fractions (LDM) from unstimulated and insulin-treated cardiomyocytes. Equal amounts of membrane proteins (20 μg) from the different fractions were laid on gels. The amount of syntaxin 1A and 1B and SNAP25 was determined by immunoblot analysis by using specific antibodies. Representative autoradiograms, obtained after various times of exposure, are shown.

both in cell surface membranes (*i.e.* fractions 23F1 and 23F2) and in intracellular membranes (Fig. 9). In cardiomyocyte membranes, syntaxin 1 and SNAP 25 were detected (Fig. 10). Both syntaxin 1A and syntaxin 1B were clearly visualized after Tris-urea SDS-PAGE gels (Fig. 10, *middle panel*). All three proteins were mainly detected in plasma membrane fractions obtained from isolated cardiomyocytes, and insulin did not cause any redistribution among membrane fractions (Fig. 10). As expected based on their membrane distribution pattern, syntaxin 1 and SNAP 25 did not colocalize with intracellular GLUT4 in cardiomyocyte membranes (data not shown).

Discussion

In this study, we have purified two different intracellular GLUT4 membrane populations by starting from insulin-sensitive and insulin-insensitive membrane fractions enriched in GLUT4 obtained from rat skeletal muscle. These two GLUT4 vesicles showed the following properties: 1) these two GLUT4 membrane populations showed a different insulin responsiveness: whereas one pool is depleted of GLUT4 in response to insulin treatment *in vivo*, a second GLUT4 pool does not respond to insulin; and 2) these two vesicle GLUT4 populations showed a different protein composition. Thus, GLUT4 vesicle immunoadsorption followed by Western blot revealed the presence of VAMP2 in the insulin-sensitive GLUT4 pool and VAMP2, cellubrevin, and SCAMPs in the insulin-insensitive GLUT4 pool. Our results suggest that VAMP2 must play an important role in GLUT4 recruitment to the cell surface in skeletal muscle, whereas SCAMPs or cellubrevin do not have a direct participation in the recruit-

ment of GLUT4. Furthermore, GLUT4 vesicle immunoadsorption followed by SDS-PAGE and silver staining showed a number of distinct bands; all the bands found in the insulin-sensitive GLUT4 pool were also detected in the insulin-insensitive population, but the reverse was not true. There were also differences in the ratio GLUT4/associated proteins in the two pools; the insulin-sensitive pool contains less associated proteins and similar abundance in GLUT4 than the insulin-insensitive pool. An important future task entails the identification of these proteins associated with the insulin-sensitive GLUT4 pool.

The precise nature of the insulin-sensitive and insulin-insensitive intracellular GLUT4 membrane pools remains unknown. We have found that all the proteins detected in the insulin-sensitive GLUT4 pool are also detected in the insulin-insensitive pool; however, the reverse is not true, and some proteins are exclusive to the insulin-insensitive GLUT4 pool. Based on the proteins associated with the insulin-insensitive GLUT4 pool, it might represent an endosomal compartment. Based on the pattern of protein composition, one possibility is that the insulin-sensitive pool derives from the insulin-insensitive GLUT4 pool. According to this hypothesis, GLUT4 would be sorted from the insulin-insensitive membrane population to the insulin-sensitive pool together with VAMP2 and a discrete number of as yet unidentified polypeptide bands. However, we have no evidence supporting the contention that GLUT4 moves from the cell surface to the insulin-insensitive population and we do not have either any direct evidence for GLUT4 being sorted from the insulin-insensitive to the insulin-sensitive pool. In any case, the model we propose shows some similarities with models recently proposed in adipocytes. Analysis of GLUT4 endocytosis and exocytosis obtained in isolated rat adipocytes predicts that GLUT4 localizes to at least two distinct intracellular compartments in adipocytes (33) and data of subcellular trafficking of both GLUT4, GLUT1, and chimeric transporters performed in 3T3-L1 adipocytes are best explained by a model postulating two intracellular pools (35). According to the predictions in adipocytes, GLUT4 is internalized into an endosomal compartment and then sorted into an insulin-recruitable compartment (35). Furthermore, our results support are also in keeping with data obtained after endosomal compartment ablation, which has been shown to reduce the amount of cellular GLUT4 in 3T3-L1 adipocytes by 40% (36).

A fundamental question is the study of the localization of the insulin-sensitive and insulin-insensitive GLUT4 pools in the muscle fiber. To tackle this issue, we are currently conducting studies of confocal microscopy using double labeling. However, this is a complex task, namely due to the inherent difficulty of assessing precisely the morphology of the muscle fiber and also due to the relatively low expression of some of the proteins, such as SCAMPs, cellubrevin, and VAMPs, which colocalize with GLUT4. In any case, it is of note to remark that, in our experimental protocol, the insulin-sensitive pool is initially obtained in a membrane fraction also yielding cell surface membranes that contain sarcolemma but not T-tubule membranes (5, 15). Furthermore, the insulin-insensitive pool is obtained in an initial membrane fraction, also yielding cell surface membranes that contain both T-tubules and sarcolemmal membranes (5, 15). Based on this, it might be postulated that the insulin-sensitive pool is close to sarcolemma (subsarcolemmal vesicles), whereas the insulin-insensitive pool might represent a GLUT4 population close to T-tubules. Based on the lack of colocalization between GLUT4 and TGN38, we suggest that these GLUT4 vesicles pools do not represent membranes from the trans-Golgi network.

GLUT4 is recruited to the cell surface in the muscle fiber in response to both insulin or exercise, and there is controversy as to whether insulin and muscle contraction cause translocation of GLUT4 from the same intracellular membrane population or from separate membrane pools. Thus, several authors have substantiated GLUT4 recruitment to the cell surface in response to insulin or muscle contraction; however, in the same studies, insulin but not exercise caused a decrease in the intracellular GLUT4 membrane population (12, 59). In contrast, other authors reported that both exercise and insulin increased cell surface GLUT4 concomitantly with decreases in intracellular GLUT4 (60, 61). An intracellular exercise-sensitive GLUT4 pool has recently been identified from rat skeletal muscle, showing a similar protein composition but differences in sedimentation coefficient compared with an insulin-sensitive GLUT4 population (62). Because the insulin-sensitive and insulin-insensitive GLUT4 pools found in our study display differences in protein composition, they are probably not identical to the exercise-sensitive pool reported by Coderre *et al.* (62).

Another important new finding of the present study is that intracellular GLUT4 vesicles obtained from skeletal muscle contain the vSNARE proteins VAMP2 and cellubrevin. We detected that cellubrevin colocalizes with only the insulin-insensitive GLUT4 pool, whereas VAMP2 is detected both in the insulin-sensitive and in the insulin-insensitive pool. Thus, more than one vSNARE protein might be involved in GLUT4 trafficking in skeletal muscle, and whereas VAMP2 might be important in insulin-induced translocation of GLUT4 to the cell surface, cellubrevin might be important in other steps in GLUT4 trafficking. The specific role of each particular vSNARE protein deserves further study, which requires the specific inhibition of cellubrevin or VAMP-2. Our results are in keeping with previous reports indicating that cellubrevin and VAMP2 colocalize with GLUT4 in intracellular membranes obtained from adipocytes (29, 30). Furthermore, it has recently been reported that cleavage of VAMP2 and cellubrevin in GLUT4-containing vesicles, by clostridial neutoxins, inhibits the translocation of GLUT4 in 3T3-L1 adipocytes (63). We also describe the presence of the t-SNARE protein SNAP-25, but not syntaxin 1A or syntaxin 1B, in skeletal muscle membranes. In all, our data suggest the involvement of the SNAP/SNARE mechanism in the cellular trafficking of GLUT4 in skeletal muscle.

In our study, we have also explored the expression of v- and t-SNAREs in membranes from isolated rat cardiomyocytes and the colocalization with intracellular GLUT4. Thus, we have found cellubrevin but not VAMP2 in GLUT4 vesicles obtained from cardiomyocytes. We also describe the presence of the t-SNARE proteins SNAP-25, syntaxin 1A and syntaxin 1B in plasma membranes from cardiomyocytes. These data further strengthen the involvement of the SNAP/

SNARE mechanism in the cellular trafficking of GLUT4 in muscle cells and suggest the existence of differences in the regulation of GLUT4 trafficking mediated by SNARE proteins when comparing skeletal and cardiac muscle. Because the presence of syntaxin 4 has been reported in skeletal muscle and L6 muscle cells (64), it is of interest to investigate which precise v-/t-SNARE interactions drive this particular vesicle pathway in each muscle type.

Acknowledgments

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