Association of Acyl-CoA Synthetase-1 with GLUT4-containing Vesicles*

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GLUT4, the glucose transporter present in insulin-sensitive tissues, resides in intracellular vesicular structures and translocates to the cell surface in response to insulin. In an attempt to identify proteins present in these structures, GLUT4-enriched vesicles prepared from rat adipocytes treated with or without insulin were prepared by sucrose velocity centrifugation and immunoabsorbed with anti-GLUT4 antibody. We report here the sequence identification by high performance liquid chromatography-ion trap mass spectrometry of a p75 protein band, long chain acyl-CoA synthetase-1, specifically present in immunoabsorbed GLUT4-containing vesicles but not in vesicles adsorbed by nonimmune serum. Acyl-CoA synthetase activity detected in GLUT4-enriched vesicles prepared by gradient centrifugation from insulin-treated adipocytes was decreased to about the same extent as GLUT4 protein. Additionally, immunoabsorbed GLUT4 vesicles were found to catalyze palmitoylation of proteins when incubated with labeled palmitate, a pathway that requires palmitate esterification with CoA. These data indicate that the insulin-sensitive membrane compartment that sequesters GLUT4 in fat cells contains long chain acyl-CoA synthetase-1 and its product fatty acyl-CoA, shown previously to be required for budding and fusion in membrane trafficking processes.

One of the most important actions of insulin in vivo is that it causes increased glucose utilization in fat and muscle tissues. It is now widely accepted that a major mechanism by which insulin mediates this effect is to increase the number of GLUT4 glucose transporters present on the plasma membrane (1–3). In the resting cell, GLUT4 appears to concentrate in small tubules and specialized vesicles clustered in the vicinity of the endosomal-Golgi network system (4, 5) while recycling through the resting cell, GLUT4 appears to concentrate in small tubules

bition of endocytosis also occurs (7). A number of proteins found to be present in GLUT4-containing membranes may relate to the mechanism of GLUT4 recycling (8). For example, insulin appears to direct phosphatidylinositol 3-kinase, known to be essential for protein sorting in yeast, to GLUT4-containing vesicles isolated from both 3T3-L1 and rat adipocytes (9). Members of the VAMP family have also been documented to colocalize with GLUT4 vesicles (10–12), and more recently syntaxin 4, VAMP2 and/or VAMP3/cellubrevin have been implicated as functional t-SNAREs for insulin-stimulated GLUT4 translocation (13). Similarities between GLUT4 vesicle trafficking and synaptic vesicle docking and fusion are suggested by the specific cleavage of vesicle proteins by toxins that can inhibit the ability of insulin to stimulate translocation of GLUT4 (14) and the apparent requirement of the small GTP-binding protein, Rab-4, for GLUT4 translocation (15, 16). In synaptic systems, a core complex of proteins binds NSF and a soluble NSF attachment protein (SNAP) to form a large 20 S complex. Subsequent hydrolysis of ATP by NSF, resulting in disruption of this complex, is thought to facilitate the membrane fusion event. NSF and the SNAP proteins have recently been shown to be highly enriched in GLUT4-containing vesicles and low density microsomes under steady state conditions (17).

In this study we present the polypeptide composition of GLUT4-containing vesicles and the identification of sequences corresponding to the long chain acyl-CoA synthetase-1 (EC 6.2.1.3). We demonstrate acyl-CoA synthetase activity to be associated with these vesicles, consistent with the proposed hypothesis (18–20) that fatty acyl-CoA plays a role in membrane budding or fusion events.

MATERIALS AND METHODS

Subcellular Fractionation and Preparation of GLUT4-enriched Vesicles—Adipocytes were isolated from the epididymal fat pads of male Sprague-Dawley rats (150–175 g) by collagenase digestion using Krebs-Ringer/Hepes, pH 7.4, supplemented with 2% bovine serum albumin and 2 mM pyruvate (7) and incubated in the absence or presence of insulin (10–10 M) for 10 min at 37 °C. The cells were immediately homogenized and subjected to differential centrifugation as described previously (21) to obtain membrane preparations. GLUT4-enriched vesicles were isolated from low density microsomes by sucrose velocity gradient centrifugation as described (9, 22). This material was resuspended in Buffer B (50 mM Hepes, 1 mM EDTA, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 10 mM NaF, 100 mM Na3VO4, 1 mM Na3P, 10 mg/ml aprotinin, and 5 mg/ml leupeptin) and represents the starting GLUT4-enriched vesicles used for all immunoabsorption experiments.

Immunoaosorption and Electrophoresis of GLUT4-containing Vesicles—For each experiment, 500–1000 μg of GLUT4-enriched vesicles were prewashed with Sepharose beads, and the supernatants were transferred to fresh tubes and incubated for 18 h with either anti-GLUT4 IgG (polyclonal 1288) or nonimmune IgG prebound to protein A-Sepharose as described previously (9). The immunoadsorbed vesicles were washed several times and then eluted in sample buffer and analyzed by SDS-PAGE1 and immunoblotting. In labeling experiments GLUT4-enriched vesicle proteins were conjugated to 125I using Bolton-Hunter reagent essentially as described by the manufacturer’s instructions. The radiolabeled GLUT4-enriched vesicle proteins were dissolved

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The abbreviations used are: NSF, N-ethylmaleimide-sensitive fusion protein; PAGE, polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography; MS, mass spectrometry; MOPS, 4-morpholinepropanesulfonic acid; IC-16, 16-iodohexadecanoic acid; LC, liquid chromatography; NI, nonimmune.

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in SDS-lysis buffer, denatured at 95 °C and then resolved by SDS-PAGE on 5–15% gradient gels. Proteins were then transferred to polyvinylidene difluoride membranes and exposed to X-AR film (Kodak).

**Sequencing**—The bands corresponding to proteins of interest were excised from two single dimensional, 10% SDS-polyacrylamide gels after being visualized by copper staining (Bio-Rad). After in gel S-carboxyamidomethylation, the band(s) were subjected to in gel endoproteinase LysC (Wako) digestion. Sequences were determined by capillary high performance liquid chromatography (HPLC) coupled to the electrospray source of a quadrupole ion trap mass spectrometer (Finnigan LCQ) as described by Nash et al. (23). The instrument was programmed to acquire successive sets of three scan modes consisting of full scan MS over the m/z range 395–1200, followed by two data-dependent scans on the most abundant ion in that full scan. These data-dependent scans allowed the automatic acquisition of a high resolution scan to determine charge state and exact mass and collisionally induced dissociation spectra for peptide sequence information. Manual interpretation of the resulting MS/MS spectra of GLUT4-associated peptides was facilitated with the database searching algorithm SEQUEST (24).

**Assay of Acyl-CoA Synthetase Activity**—An acyl-CoA synthetase activity was measured at 30 °C using exogenous fatty acid as substrate by a colorimetric-spectrophotometric method (25). The assay involves initial conversion of the fatty acids to their CoA derivative by the endogenous acyl-CoA synthetase with the addition of CoA (500 μM) and ATP (0.5 mM) in 1 ml of extraction buffer (150 mM MOPS, pH 7.1, 12 mM MgCl2, 150 mM KCl, 1 mM dithiothreitol, 1% methanol, 0.1% Triton X-100). The acyl-CoA product is detected by a series of reactions (acyl-CoA oxidase, 0.2 units/ml SDS, 2% dithiothreitol, 1% catalase, 2 units/ml, and 0.6% 4-amino-3-hydrazino-5-mercapto-1,2,4-triazole), which upon oxidation by IO3- (0.375%) gives a purple heteroaromatic dye that can be detected at 550 nm. All assays were carried out within the linear range with respect to reaction time, and the initial rate of reaction was proportional to the amount of enzyme added.

**Cell-free Palmitoylation Assay**—Fractions were mixed with a cell-free palmitoylation assay and the reaction was started with addition of 0.5 mM ATP, 500 mM CoA, 0.5 mM palmitic acid (10 μCi/0.5 mM, hot to cold). The 16-carboxyacetic acid (16CIC) (Dr. Marilyn Resh, Rockefeller University, New York, NY) was labeled as outlined in Peseckis et al. (26). The reaction was incubated at 30 °C for 20 min and quenched by adding 4 × SDS sample buffer and heating the samples to 100 °C. Palmitoylation of IgG was performed by incubating the purified anti-GLUT4 IgG fraction (4 mg/ml) diluted in AC buffer, acyl-CoA synthetase (20–40 milliunits, Boehringer Mannheim), 200 mM CoA, 2 mM ATP, and [125I]-iodohexadecanoic acid (IC-16) (Dr. Marilyn Resh, Rockefeller University, New York, NY) was labeled as outlined in Peseckis et al. (26). The reaction was stopped by the addition of sample buffer, and the proteins were resolved on 10% SDS-polyacrylamide gels, dried between sheets of cellophane, and then exposed to film at 80 °C with intensifying screens.

**Electron Microscopy**—GLUT4-enriched vesicles were fixed in a final concentration of 2% paraformaldehyde in phosphate-buffered saline, adsorbed to Formvar-coated nickel grids (300 mesh, Ladd Company) and processed for double labeling as outlined in Martin et al. (27). Grids were postfixed with 10 μl of primary antibody diluted in 1% bovine serum albumin/phosphate-buffered saline as follows: anti-GLUT4 (4 μg/ml) and anti-vp165 IgG (5 μg/ml). After incubation with each primary antibody, grids were labeled with either 5 or 15 nm gold particles conjugated to the secondary antibody (goat anti-rabbit). Grains were stained with 1% uranyl acetate, dried, and viewed using a transmission electron microscope (JOEL 100CX, 80 kV short beam).

**RESULTS AND DISCUSSION**

GLUT4-enriched vesicles were prepared by a sucrose velocity gradient sedimentation technique previously reported (22), and all fractions taken from the gradient were analyzed by Western blot with specific antibodies for the presence of GLUT4 protein. Fractions 8 to 18 were pooled, and recovery of GLUT4 protein was consistently 5% of the low density microsomal fraction. Analysis of these pooled fractions by double label immunoelectron microscopy revealed the presence of intact rounded, vesicular-like structures (50–80 nm) that intensely labeled with anti-GLUT4 as well as antibodies against vp165, an amineopeptidase that exhibits nearly 100% overlap with GLUT4 in cellular distribution (28, 29) (Fig. 1). Importantly, virtually all vesicles that were labeled by anti-GLUT4 also labeled with anti-vp165, indicating colocalization of GLUT4 and vp165 in the same vesicles as previously proposed (8–10). These data also confirm the utility of gradient sedimentation as a means for partially purifying GLUT4-enriched vesicles from fat cell membranes.

To determine whether additional protein components are specific to these vesicles, equal amounts of GLUT4-enriched vesicles were re-isolated and immunoadsorbed with an antibody raised against the COOH terminus of GLUT4 or an equal amount of nonimmune IgG. Fig. 2 depicts the SDS-PAGE profile of the immunoadsorbed vesicles derived from adipocytes treated for 10 min with or without insulin. Proteins (designated a–i in Fig. 2) that are present in vesicles immunoadsorbed with anti-GLUT4 but are absent or present at lower levels in vesicles immunoadsorbed with nonimmune IgG migrate at the molecular masses of 165, 120, 110, 92, 75, 67, 48, 36, and 35 kDa. Specific decreases in these same protein bands are observed in response to insulin treatment of intact cells prior to fractionation (Fig. 2, lane 1 versus lane 3). The identification of the same proteins by the two different approaches (anti-GLUT4 versus anti-nonimmune IgG adsorption and control versus insulin treatment) provides high confidence that these proteins actually colocalize to GLUT4-containing vesicles.

To identify proteins designated in the profile shown in Fig. 2, the immunoadsorption procedure was scaled up to obtain proteins in sufficient quantities for protein microsequencing. The eluted proteins were analyzed by copper and silver stain (Fig. 3), and the protein bands corresponding to p165 and p75 were excised and subjected to proteolytic cleavage followed by online LC/MS/MS sequencing by ion trap mass spectrometry. Six peptide sequences (NAATTQWEPLAAR, OSVTTSLQLQDTR, KGTELLLQQER, LLGMSFMNR, LPTAIIPQR, and GFPLPE) were detected using protein A conjugated to different size gold particles. Grids were coated with 1% uranyl acetate, dried, and viewed using a transmission electron microscope (JOEL 100CX, 80 kV short beam).

**FIG. 1.** GLUT4 and vp165 colocalization in GLUT4-enriched vesicles preparations. Shown are electron micrographs of GLUT4-enriched vesicles from rat fat. Vesicles prepared by gradient centrifugation were fixed in 2% paraformaldehyde and adsorbed to Formvar-coated nickel grids. The grids were labeled with specific antibodies that were detected using protein A conjugated to different size gold particles. a, colocalization of GLUT4 (5 nm, small arrow) and vp165 (15 nm, large arrowheads). b, colocalization of GLUT4 (15 nm, small arrows) and vp165 (5 nm, large arrowheads).
GLUT4-enriched vesicles from adipocytes for acyl-CoA synthetase activity using the exogenous substrate palmitic acid revealed significant activity (672 ± 690 pmol/min/mg) that was inhibited by Triacsin-C, a known competitive inhibitor of acyl-CoA synthetase (Fig. 4b). Insulin treatment of intact fat cells significantly reduced the total acyl-CoA synthetase activity for GLUT4-enriched vesicles recovered from the velocity gradient fractions when both palmitic (16:0) and oleic (18:1) acids were used as substrates. No corresponding increase in acyl-CoA synthetase activity in other membrane or cytosolic fractions could be detected in response to insulin (not illustrated), but such increases may be too small to detect. Importantly, the decrease in activity observed in GLUT4-containing vesicles was similar to that observed for GLUT4 and vp165 protein (Fig. 4a), suggesting that the amount of acyl-CoA synthetase is decreased in GLUT4-enriched vesicle preparations in response to insulin. These data are consistent with the conclusion that acyl-CoA synthetase is a component of the insulin-sensitive membrane compartment.

To confirm that acyl-CoA synthetase is present in GLUT4-enriched vesicles, we used a sensitive assay that couples the fatty acyl esterification reaction to the acylation of an artificial protein substrate, in this case, rat IgG. In this reaction acyl-CoA synthetase catalyzes the esterification of the 125I-labeled palmitic acid analog, [125I]IC-16, to palmitoyl-CoA, providing a labeled acyl group that can be transferred to proteins even in the absence of any specific palmitoyl-transferase. Results from a control reaction of purified rat IgG with CoA, ATP, [125I]IC-16, and acyl-CoA synthetase shows the incorporation of the labeled fatty acyl group into the heavy chain of IgG (Fig. 5a). As indicated in the results from this experiment, the reaction is dose-dependent for both active acyl-CoA synthetase and substrate. To assess the acyl-CoA synthetase activity associated with purified GLUT4-containing vesicles, equal amounts of

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**Fig. 2.** Resident proteins in immunoadsorbed GLUT4-containing vesicles. Purified GLUT4-containing vesicles were immunoadsorbed from 125I-labeled GLUT4-enriched vesicles (prepared by gradient centrifugation) with anti-GLUT4 antibody (lanes 1 and 3) or NI IgG (lanes 2 and 4). Prior to membrane preparation, cells were treated without (lanes 1 and 2) or with insulin (lanes 3 and 4). The purified GLUT4-containing vesicles were analyzed by SDS-PAGE on 5–15% gradient gels, transferred to polyvinylidene difluoride filters, and exposed to Kodak X-AR film for approximately 48 h.

**Fig. 3.** Sequences of protein peptides isolated from GLUT4-containing vesicles reveal the presence of long chain acyl-CoA synthetase-1. Rat adipocytes (~100 g) were isolated and homogenized, and a GLUT4-enriched vesicle population was prepared as described under “Materials and Methods.” This fraction was immunoadsorbed with either anti-GLUT4 antibody or NI IgG, eluted, separated by SDS-PAGE, and visualized with copper stain. Approximately 15 pmol of p165 and 5 pmol of p75 were excised and subjected to lysyl endoproteinase digestion, and 10% was consumed for sequencing by LC/MS/MS. The sequence of one peptide from p165 (a) is shown aligned to the aminopeptidase vp165 (see text for other peptides), and peptides from p75 (c) aligned to long chain acyl-CoA synthetase-1 (ACS-1).
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Previous studies designed to identify molecular elements within the intracellular GLUT4-enriched membranes have yielded the identities of a number of proteins known to be involved in membrane trafficking of other cellular systems such as synaptic vesicle exocytosis and transport between Golgi membranes. This is also the case for the present finding that long chain acyl-CoA synthetase is a component of insulin-sensitive GLUT4-containing vesicles (Figs. 3–5), because long chain fatty acyl-CoA is required for Golgi membrane fusion and fission events in vitro (20). Thus, it seems likely that the basic elements of GLUT4 recycling to the plasma membrane involve mechanisms common to other better characterized membrane trafficking systems. An important goal of future studies is to determine the molecular basis of fatty acyl-CoA function in GLUT4 recycling and other membrane trafficking pathways.

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