Glut4 Storage Vesicles without Glut4: Transcriptional Regulation of Insulin-Dependent Vesicular Traffic

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Two families of transcription factors that play a major role in the development of adipocytes are the CCAAT/enhancer-binding proteins (C/EBPs) and the peroxisome proliferator-activated receptors (PPARs), in particular PPAR γ . Ectopic expression of either C/EBP α or PPAR γ in NIH 3T3 fibroblasts results in the conversion of these cells to adipocyte-like cells replete with fat droplets. NIH 3T3 cells ectopically expressing C/EBP α (NIH-C/EBP α) differentiate into adipocytes and exhibit insulin-stimulated glucose uptake, whereas NIH 3T3 cells ectopically expressing PPAR γ (NIH-PPAR γ) differentiate but do not exhibit any insulin-stimulated glucose uptake, nor do they express any C/EBP α . The reason for the lack of insulin-responsive glucose uptake in the NIH-PPAR γ cells is their virtual lack of the insulin-responsive glucose transporter, Glut4. The NIH-PPAR γ cells express functionally active components of the insulin receptor-signaling pathway (the insulin receptor, IRS-1, phosphatidylinositol 3-kinase, and Akt2) at levels comparable to those in responsive cell lines. They also express components of the insulin-sensitive vesicular transport machinery, namely, VAMP2, syntaxin-4, and IRAP, the last of these being the other marker of insulin-regulated vesicular traffic along with Glut4. Interestingly, the NIH-PPAR γ cells show normal insulin-dependent translocation of IRAP and form an insulin-responsive vesicular compartment as assessed by cell surface biotinylation and sucrose velocity gradient analysis, respectively. Moreover, expression of a Glut4-myc construct in the NIH-PPAR γ cells results in its insulin-dependent translocation to the plasma membrane as assessed by immunofluorescence and Western blot analysis. Based on these data, we conclude that major role of C/EBP α in the context of the NIH-PPAR γ cells is to regulate Glut4 expression. The differentiated cells possess a large insulin-sensitive vesicular compartment with negligible Glut4, and Glut4 translocation can be reconstituted on expression of this transporter.

Adipose tissue plays a central role in the regulation of energy balance by virtue of its ability to store fuel in the form of triacylglycerides, to provide fuel in the form of fatty acids, and to secrete a number of hormones and cytokines (14). The cytokines act peripherally and in the brain to maintain organismal energy balance and insulin sensitivity (14). The dysregulation of adipocyte insulin action has been proposed to be a critical event in the development of the various pathologies originating from the metabolic syndrome (5). A principal action of insulin in adipocytes is the stimulation of glucose transport as a result of translocation to the cell surface of the muscle/adipocyte glucose transporter, Glut4 (8). The transported glucose is metabolized to form the glycerol backbone for triglyceride storage, and the adipocyte-specific ablation in mice of Glut4 expression leads to insulin resistance (1). Despite the critical function of adipocyte glucose transport, many of the details by which adipocytes (and muscle) form a pathway of insulin-sensitive Glut4 trafficking remain unknown (53).

The development and maturation of insulin-sensitive adipocytes is regulated in a coordinate manner by a number of transcription factors including peroxisome proliferator-activated receptor γ (PPAR γ) and several members of the CCAAT/enhancer-binding proteins (C/EBPs) (10, 46, 55). In the course of differentiation of 3T3L1 fibroblasts into adipocytes, C/EBP β and C/EBP δ are expressed transiently in that order and their

levels peak early in the time course of differentiation (67). This is followed by the virtual simultaneous expression of C/EBP α and PPAR γ on day 2 of the differentiation process, and this expression is sustained through day 8 (67). Glut4 expression is observed on days 4 to 5 and continues to increase through day 8, when maximal insulin-sensitive glucose transport is observed (6, 13). Knocking out either PPAR γ (4, 49) or C/EBP α (11, 63) genes in mice blocks the full development of adipocytes.

In agreement with the knockout results are gain-of-function experiments showing that the ectopic expression of either PPAR γ (61), C/EBP α (16), or C/EBP β (66) in fibroblasts activates the adipogenic program and converts these cells into adipocytes. However, the acquisition of the adipocyte phenotype, as determined by accumulation of lipid droplets in the cell and expression of fat-specific proteins such as the fatty acid-binding protein aP2 (18), does not guarantee that the cells will possess robust insulin-stimulated glucose uptake; rather, this process requires C/EBP α expression. Thus, NIH 3T3 fibroblasts that ectopically express PPAR γ (NIH-PPAR γ) differentiate into adipocytes but lack C/EBP α expression and show minimal Glut4 expression and, consequently, an insignificant increment of insulin-stimulated glucose uptake (12, 20). PPAR γ ectopically expressed in mouse embryo fibroblasts derived from C/EBP α knockout mice also results in adipocyte conversion without insulin-stimulated glucose uptake (65). In the latter case, insulin receptor expression and downstream signaling activity were found to be significantly decreased, and it was concluded that this was the reason for the lack of insulin responsiveness.

Thus, the insulin-stimulated glucose uptake characteristic of fat and muscle tissue requires an intact insulin-signaling

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pathway as well as the target of this signaling, a large pool of membrane vesicles enriched in Glut4 (53). This vesicular pool, variously called glucose transporter storage vesicles (GSVs) or insulin-responsive vesicles (IRVs), forms early during adipocyte differentiation (13) and, in addition to Glut4, is characterized by the abundant presence of a leucyl aminopeptidase (insulin-responsive aminopeptidase [IRAP]) that traffics in a manner similar or identical to that of Glut4 (32, 38, 42, 44, 50). Both Glut4 (36) and IRAP (37) knockout animals are viable, and the question has been raised whether GSVs/IRVs need to have Glut4 (or IRAP) expression to exist. In the experiments described in this report, we used NIH 3T3 fibroblasts ectopically expressing either PPAR γ or C/EBP α to examine parameters of insulin-stimulated vesicular trafficking. The former express minimal amounts of Glut4, but during their differentiation, both cell lines develop a robust pool of intracellular vesicles containing IRAP, and these translocate to the cell surface in response to insulin.

MATERIALS AND METHODS

Materials. Dexamethasone, 3-isobutyl-1-methylxanthine, insulin, benzamide, microcystin, sodium fluoride, sodium orthovanadate, puromycin, wortmannin, 2-deoxyglucose, fetal bovine serum (Australian origin), donkey serum, mouse immunoglobulin G (IgG) antibody, Oil Red O, and digitonin were purchased from Sigma (St. Louis, Mo.). Aprotinin, leupeptin, and pepstatin A were obtained from American Bioanalytical (Natick, Mass.). Calf serum was purchased from Life Technologies (Gaithersburg, Md.), and Dulbecco's modified Eagle's medium (DMEM) was from Mediatech, Inc. (Herndon, Va.). 2-[3 H]deoxyglucose was purchased from New England Nuclear (Boston, Mass.), FuGENE 6 transfection reagent was purchased from Roche (Indianapolis, Ind.), and EZ-Link sulfo-NHS-SS-biotin and ImmunoPure immobilized streptavidin-agarose beads were purchased from Pierce (Rockford, Ill.). Geneticin (G418 sulfate) was purchased from Invitrogen (Carlsbad, Calif.), and protein A-agarose was from Upstate Cell Signaling Solutions (Lake Placid, N.Y.). Troglitazone was a kind gift from John Johnson (Parke-Davis/Warner Lambert, Ann Arbor, Mich.). The pLNCX2 Glut4-myc construct was a kind gift from Kostya Kandror, Department of Biochemistry, Boston University School of Medicine, Boston, Mass. The pCLeco DNA and HEK293 cells were kind gifts from Domenico Tortorella, Department of Microbiology, Mount Sinai School of Medicine, New York, N.Y.

Antibodies. In this study we used the monoclonal anti-Glut4 antibody IF8 (26), goat polyclonal anti-Glut4 antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, Calif.), affinity-purified anti-IRAP serum (Quality Control Biochemicals/Bio-source), polyclonal anti-insulin receptor antibody (Transduction Laboratories, Lexington, Ky.), a sheep polyclonal anti-phosphoAkt (pSer473) antibody, polyclonal anti-phosphatidylinositol 3-kinase (anti-PI3-kinase) antibody (Upstate Biotech Inc., Lake Placid, N.Y.), polyclonal anti-Syntaxin4 and monoclonal anti-VAMP2 antibodies (Synaptic Systems), monoclonal anti-TfR antibody H68 (Zymed Laboratories, South San Francisco, Calif.), monoclonal anti-Myc-Tag antibody 9B11 (Cell Signaling Technologies, Beverly, Mass.), and monoclonal anti-PPAR γ and polyclonal anti-C/EBP α antibodies (Santa Cruz Biotechnology). The following antibodies were generous gifts: polyclonal anti-Glut1 antibody (C. Carter-Su, University of Michigan, Ann Arbor, Mich.), polyclonal anti-IRS-1 serum (M. Gibbs, Department of Cardiovascular and Metabolic Diseases, Pfizer Inc., Groton, Conn.), and polyclonal anti-Akt2 antibody (M. J. Birnbaum, Howard Hughes Medical Institute, University of Pennsylvania, Philadelphia, Pa.).

Cell culture. Murine NIH 3T3 fibroblasts ectopically expressing either PPAR γ or C/EBP α via retroviral infection were cultured, maintained, and differentiated as described previously (12). Briefly, cells were plated and grown in DMEM containing 10% fetal bovine serum (FBS) and 2.0 μ g of puromycin per ml. At 2 days postconfluence (day 0), the cells were induced by changing the medium to DMEM containing 10% FBS, 0.5 mM 3-isobutyl-1-methylxanthine, 1 μ M dexamethasone, 1.7 μ M insulin, and 5 μ M troglitazone. After 48 h, the induction medium was removed and cells were maintained in DMEM containing 10% FBS and 5 μ M troglitazone.

Oil Red O Staining. Oil Red O staining was performed as described by Green and Kehinde (19) with minor modifications. The cells were photographed using an Olympus IX70 microscope.

Retroviral infections. Retroviral supernatant was generated using HEK293 cells, which were transiently transfected using FuGENE 6 transfection reagent. FuGENE 6 reagent was incubated with warm DMEM for 5 min at room temperature. Next, the DNA was introduced; 6 μ g of retroviral vector pLNCX2 containing Glut4-myc along with 6 μ g of the pCLeco replication-incompetent helper plasmid (47). The FuGENE-DNA mix was incubated for 30 min at room temperature and then was dropped into a 10-cm dish of HEK293 cells containing 10 ml of 10% FBS-DMEM, approximately 50 to 60% confluent. After 24 h, the medium was changed to 6 ml of 10% FBS-DMEM, and this was left for an additional 24 h, after which the viral supernatant was filtered through a 0.45- μ m-pore-size cellulose acetate filter and 3 ml was added to target cells (NIH-PPAR γ) along with 10 μ g of Polybrene per ml. The viral medium was left on the cells for 4 h at 37°C, an additional 3 ml of 10% FBS was added, and the cells were left overnight. After 72 h, selection with G418 (150 μ g/ml) began and was continued for 2 weeks.

Preparation of whole-cell extracts. At the indicated times, cultured cells grown in 6-cm dishes were rinsed three times with phosphate-buffered saline (PBS) and then harvested in ice-cold buffer containing 50 mM Tris (pH 7.4), 100 mM NaCl, 1% sodium deoxycholate, 4% Nonidet P-40, (NP-40), 0.4% sodium dodecyl sulfate (SDS), 1 μ M pepstatin, 1 μ M aprotinin, and 10 μ M leupeptin. Lysates were vortexed and stored at -20°C for the duration of the collection for the various days. When ready to be analyzed, samples were thawed, vortexed, and centrifuged for 30 minutes at 16,000 $\times$ g in a microfuge at 4°C. The supernatants were collected, and the protein content was determined using the bicinchoninic acid (BCA) kit (Pierce).

2-[3 H]deoxyglucose uptake. The 2-[3 H]deoxyglucose uptake assay was performed with six-well plates as described previously (13, 56) with minor modifications. Briefly, cells were washed twice with warm DMEM and then serum starved for 2 h in DMEM at 37°C. The cells were treated with 100 nM insulin or carrier for 15 min. They were washed twice with warm KRH buffer (121 mM NaCl, 4.9 mM KCl, 1.2 mM MgSO $_4$, 0.33 mM CaCl $_2$, 12 mM HEPES [pH 7.4]) without glucose, and then the glucose uptake was performed for 7.5 min. The concentration of 2-deoxyglucose was 2 mM, with 1 μ Ci of 2-[3 H]deoxyglucose/ml. The reaction was terminated by washing the cells three times with ice-cold KRH buffer containing 25 mM glucose. The cells were then solubilized in 1 ml of a buffered digitonin solution (0.25 M mannitol, 17 mM morpholine propane sulfonic acid [MOPS] [pH 7.4], 2.5 mM EDTA, 8 mg of digitonin per ml). Under these conditions, hexose uptake was linear for at least 30 min. Measurements were made in duplicate and corrected for specific activity and non-specific uptake (as determined in the presence of 5 μ M cytochalasin B), which was less than 10% of the total uptake. The protein concentration was determined using the Bio-Rad protein assay kit and was used to normalize counts.

Gel electrophoresis and immunoblotting. Proteins were separated by SDS-polyacrylamide gel electrophoresis (PAGE) (acrylamide from National Diagnostics) as described by Laemmli (39) and electrophoretically transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad) in 25 mM Tris-192 mM glycine. The membranes were then blocked with 10% nonfat dry milk in PBS for 1 h at room temperature and incubated with the primary antibodies described above. Horseradish peroxidase-conjugated secondary antibodies (Sigma) and either an ECL substrate kit (New England Nuclear) or Super Signal West Femto maximum-sensitivity substrate kit (Pierce) was used for detection.

Biotinylation assay for translocation. IRAP translocation to the plasma membrane was assessed by a vectorial biotinylation method essentially as described previously (32, 58). Cells were grown to confluence in 6-cm plates and induced to differentiate. On day 8 or 9 of differentiation, the cells were washed twice and serum starved for 2 h in DMEM at 37°C. They were then preincubated for 40 min with 1 μ M Wortmannin (or 0.1% dimethyl sulfoxide) in DMEM. The cells were then washed twice with warm KRPH buffer (128 mM NaCl, 4.7 mM KCl, 1.25 mM CaCl $_2$, 1.25 mM MgSO $_4$, 5 mM Na $_2$ HPO $_4$, 20 mM HEPES [pH 7.4]) and treated with 100 nM insulin or carrier while still in the presence of 1 μ M wortmannin. During the last 2 min of the insulin treatment, a cleavable, cell-impermeable biotin analogue, EZ-Link-sulfo-NHS-SS-biotin, was added to the cells to a final concentration of 1 mM. The reaction was terminated by washing the cells three times in ice-cold quenching buffer (150 mM NaCl, 20 mM Tris-HCl, 25 mM ethanolamine [pH 7.4]) and incubating them for 5 min on ice. The cells were then washed once with ice-cold PBS and incubated with 2 mM *N*-ethylmaleimide in PBS for 3 min. They were lysed in 500 μ l of solubilization buffer containing protease inhibitors (1% Triton, 150 mM NaCl, 20 mM Tris-HCl [pH 7.4], 1 μ M aprotinin, 1 μ M pepstatin, 10 μ M leupeptin) and *N*-ethylmaleimide. The lysates were vortexed, incubated on ice for 15 min, and centrifuged at 16,000 $\times$ g for 30 min at 4°C. The protein concentration of the supernatants was determined using the BCA assay (Pierce). Protein (400 μ g) was adjusted to 400 μ l with solubilization buffer and incubated overnight with 50 μ l

of streptavidin-agarose beads, which had previously been washed with 1 ml of solubilization buffer. The following day, supernatants were collected and the beads were washed four times with 1 ml of solubilization buffer. Proteins bound to the streptavidin-agarose beads were eluted in 200 μ l of Laemmli sample buffer containing 5% β -mercaptoethanol (American Bioanalytical). Following SDS-PAGE, the proteins were electrophoretically transferred to PVDF membranes and Western blot analyses were performed using the antibodies described above. Images were scanned and quantitation was performed using National Institutes of Health Image 1.63 software. The values obtained were used to determine the mean and standard error based on three independent experiments.

Cell fractionation. Eight 10-cm dishes of NIH-PPAR γ adipocytes were used per condition. Before being harvested, the cells were washed twice with warm serum-free DMEM and then serum starved for 2 h at 37°C in DMEM. The cells were then treated with either 100 nM insulin or carrier in DMEM for 30 min at 37°C. KCN was added for 5 min at a final concentration of 0.2 mM. The cells were then washed twice with warm buffer A without EDTA (250 mM sucrose, 20 mM HEPES [pH 7.4], 1 μ M aprotinin, 1 μ M pepstatin, 10 μ M leupeptin, 5 mM benzamide). They were then harvested in 2 ml of ice-cold buffer A with 1 mM EDTA and homogenized using a Potter-Elvehjem Teflon pestle. Total membranes were fractionated by differential centrifugation into plasma membrane, heavy microsomes, light microsomes (LM), and a nuclear and mitochondrial fraction, as previously described (54, 56) with one minor modification, in which the first 90-min high-speed centrifugation step to pellet total membranes was omitted. These fractions were resuspended in PBS containing the same standard mixture of protease inhibitors present in the first buffer. The protein content was determined using a BCA kit (Pierce).

Sedimentation of LM in sucrose velocity gradients. A 750- μ g quantity of LM, resuspended in PBS with protease inhibitors, was loaded onto a 4.6-ml 10 to 30% (wt/wt) continuous sucrose gradient and centrifuged at 277,000 $\times$ g in a Beckman Instruments (Palo Alto, Calif.) SW-55.1 rotor for 55 min at 4°C as described previously (13). The sucrose gradients were prepared in a buffered solution composed of 20 mM HEPES (pH 7.4) and 1 mM EDTA. Membranes from the gradients were collected in 30 to 31 fractions starting from the bottom of the tubes. The protein profile was determined using the BCA kit (Pierce). The positions of Glut1, Glut4, and IRAP were determined by Western blot analysis.

Immunoadsorption of Glut4-containing vesicles. Protein A-purified IF8 antibody and nonspecific mouse IgG (Sigma) were each coupled to Tris-acryl beads (Reacti-Gel GF 2000; Pierce) at 1.0 mg of antibody/ml of resin according as specified by the manufacturer (34, 69). Prior to use, the antibody-coupled beads were saturated with 1% bovine serum albumin in PBS for 30 min at room temperature, and then washed three times with cold PBS. LM (200 μ g in PBS with protease inhibitors) from basal and insulin-treated NIH-PPAR γ and NIH-C/EBP α adipocytes were incubated with 10, 25, or 50 μ l of IF8-coupled beads or with 50 μ l of nonspecific antibody-coupled beads overnight at 4°C with mixing. The beads were washed three times with cold PBS and then incubated with 1% Triton X-100 in PBS for 2 h at 4°C. They were then washed three times with the 1% Triton X-100 solution, and the remaining protein was eluted with nonreducing Laemmli sample buffer.

Immunofluorescence. NIH-PPAR γ cells ectopically expressing Glut4-myc (7) subcloned into the retroviral vector pLNCX2 (Clontech) (NIH-PPAR γ + Glut4-myc), were grown on two-well chamber slides (Lab-Tek) that were treated with a fibronectin solution (20 μ g/ml) (Sigma) overnight at 4°C. The cells were grown to confluence and were induced to differentiate 2 days postconfluence as described above. On day 10 of differentiation, the cells were washed twice with warm DMEM and then serum starved for 4 h. They were treated with 100 nM insulin or carrier for 30 min. They were then washed once with PBS and fixed with 3.7% formaldehyde in PBS for 20 min at room temperature. They were washed twice more with PBS and then incubated overnight in PBS at 4°C. The following day, they were blocked for 1 h at room temperature in PBS solution containing 5% donkey serum (Sigma) and 5% bovine serum albumin (American Bioanalytical). The fixed cells were then washed three times with PBS and incubated for 1 h with monoclonal anti-Myc-Tag 9B11 antibody at a dilution of 1/250 in the blocking solution at room temperature. Four more washes with PBS were then performed, and the cells were covered in aluminum foil and incubated for 1 h at room temperature with an anti-mouse Cy3 secondary antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, Pa.) at a dilution of 1/1,000. Again, the cells were washed four times with PBS and then mounted with a glycerol-PBS solution (component A of the SlowFade antifade kit from Molecular Probes, Eugene, Oreg.). The stained cells were observed with a Zeiss Axiovert 200M microscope equipped with a Hamamatsu Photonics K.K. camera for standard immunofluorescence.

Immunoprecipitation. Cells were grown to confluence and induced to differentiate 2 days postconfluence as described above. On day 10 of differentiation,

the cells were washed twice with warm DMEM, serum starved for 4 h, and stimulated with 100 nM insulin or carrier in DMEM. They were then treated with either anti-Myc-Tag antibody (9B11) at a dilution of 1/1,000 or mouse IgG overnight at 4°C in PBS. Following antibody incubation, the cells were washed three times with ice-cold PBS and then lysed in solubilization buffer (20 mM Tris-HCl, 150 mM NaCl, 1% Triton X-100 [pH 7.4]) containing protease inhibitors (1 μ M aprotinin, 10 μ M leupeptin, and 1 μ M pepstatin). All lysates were vortexed and centrifuged at 16,000 $\times$ g for 20 min at 4°C, supernatants were collected, and protein concentrations were determined using the BCA kit (Pierce). Protein (200 μ g) was adjusted to 400 μ l with solubilization buffer and incubated with 50 μ l of protein A-agarose beads for 3 h at 4°C. Supernatants were collected, and the beads were washed four times with 1 ml of solubilization buffer. Proteins bound to the protein A-agarose beads were eluted in 200 μ l of nonreducing Laemmli sample buffer. Following SDS-PAGE, the proteins were electrophoretically transferred to PVDF membranes and Western blot analyses were performed using either anti-Myc-Tag 9B11 antibody or goat anti-Glut4 antibody. Images were scanned and quantitation was performed using NIH Image 1.63 software. The values obtained were used to determine the mean and standard error based on three independent experiments.

RESULTS

We have previously shown that NIH 3T3 fibroblasts converted to adipocytes by C/EBP α expression have insulin-responsive glucose uptake and those expressing PPAR γ do not (12). However, a new independent isolate of the NIH-PPAR γ -infected cells (the C/EBP α cells are the same) was used in the present study, and it was therefore necessary to characterize them with regard to glucose uptake (Fig. 1A), differentiation (Fig. 1C), and protein expression profiles (Fig. 1B and 2). As shown in Fig. 1A and in confirmation of our previous results (12), the NIH-PPAR γ cells were unresponsive to insulin in terms of insulin-stimulated glucose uptake whereas the NIH-C/EBP α cells exhibited a relatively robust insulin response. As was the case for 3T3-L1 adipocytes (13), the NIH-C/EBP α adipocytes showed a clear insulin response on day 4 of differentiation when Glut4 expression was minimal (Fig. 2A). The NIH-PPAR γ adipocytes did not show this insulin response, but they did demonstrate an almost identical drop in basal activity on day 4 of differentiation, an event that also occurred in 3T3L1 adipocytes.

We also verified that the NIH 3T3 cells that were used in the present study, and that were infected and converted to adipocytes by retroviral infection with PPAR γ expressed undetectable levels of C/EBP α (Fig. 1B), as we previously demonstrated (12), although, as is the case in all adipocyte cell lines, there is cell-to-cell variation in lipid content and the NIH-PPAR γ cells have about 60% of the lipid compared to cells converted to adipocytes by C/EBP α expression (Fig. 1C). This is most probably due to decreased expression of adipogenic enzymes. We examined the expression levels of a panel of genes involved in lipid metabolism (perilipin, adipocyte fatty acid binding protein, hormone-sensitive lipase, fatty acid synthase, acetyl coenzyme A carboxylase, and others) (D. N. Gross and P. F. Pilch, unpublished data) and found that they are expressed in the NIH-PPAR γ adipocytes at about 50 to 75% of the level seen in the NIH-C/EBP α adipocytes.

The lack of insulin-stimulated glucose uptake in the NIH-PPAR γ adipocytes could be due to a deficiency of the components required for the insulin-signaling pathway, a lack of Glut4, or a deficiency of both. Therefore, we compared the expression of some relevant proteins in the two cell lines, as shown in Fig. 2. Glut4 expression was minimal in the NIH-PPAR γ cells, at approximately 2 to 5% of that seen in the

FIG. 1. Insulin-responsive 2-deoxyglucose uptake in NIH-C/EBP α and NIH-PPAR γ cells. (A) On the indicated days after induction of differentiation, cells were serum starved for 2 h and then treated with 100 nM insulin (+) for 15 min at 37°C or left untreated (-). 2-[3 H]deoxyglucose uptake was then determined as described in Materials and Methods. Each data point is the average of duplicate determinations, and the average value of three independent experiments with standard error is depicted. (B) Whole-cell extracts were prepared on the indicated days of differentiation and subjected to SDS-PAGE and Western blotting to determine the protein expression of C/EBP α . Detection was carried out with HRP-conjugated secondary antibody and chemiluminescence. (C) Cells were grown to confluence in 6-cm dishes, fixed with paraformaldehyde, stained with Oil Red O on the indicated days of differentiation, and then photographed using an Olympus IX70 microscope.

FIG. 2. Protein expression profile of differentiating NIH-C/EBP α and NIH-PPAR γ cells. On the indicated days after induction, whole-cell extracts were prepared and equal amounts of protein (200 μ g) were resolved by SDS-PAGE (10 or 15% polyacrylamide) and Western blotting, (A) Glut4 vesicle component proteins (IRAP, Glut4, and Glut1). (D) Proteins involved in insulin-dependent signaling (insulin receptor, IRS-1, PI3-kinase, and Akt2) and Glut4 vesicle trafficking (Syntaxin-4 and VAMP2). Detection was with HRP-conjugated secondary antibodies and chemiluminescence. (B) The relative amounts of Glut4, Glut1, and IRAP were quantified using a computing densitometer, and the results are shown as a graph. (C) Relative levels of IRAP protein expression in 3T3L1, NIH-PPAR γ , and NIH-C/EBP α adipocytes (day 10).

C/EBP α expressing cells (Fig. 2A). In contrast, IRAP expression levels were slightly higher in the NIH-PPAR γ adipocytes than in the C/EBP α -expressing cells and were comparable to those in 3T3L1 cells (Fig. 2C). Glut1 levels decreased as differentiation proceeded in the NIH-PPAR γ cells, a pattern similar to that observed in 3T3L1 cells (13). The NIH-PPAR γ cells, however, showed a more extensive decrease in Glut1 expression on day 4 than did the 3T3L1 cells. In contrast, Glut1 expression increased throughout differentiation in the NIH-C/EBP α cells and reached much higher levels than those seen in

FIG. 3. Insulin receptor signaling in NIH-PPAR γ adipocytes. On day 8 of differentiation, cells were serum starved for 2 h, incubated with 1 μ M wortmannin for 40 min or left untreated, and treated with 100 nM insulin (+) or carrier (–) for the times indicated while still in the presence of wortmannin. The cells were lysed in a solubilization buffer containing both protease and phosphatase inhibitors. Whole-cell extract (200 μ g) was resolved by SDS-PAGE (10% polyacrylamide), and samples were subjected to Western blotting with anti-phospho (Ser473)-Akt or anti-Akt2 antibodies. Detection was as described in the legends to Fig. 1 and 2.

the NIH-PPAR γ -expressing adipocytes (Fig. 2A and B). This increase in expression could also account for the increased basal glucose uptake seen on day 8 compared with day 4 in the NIH-C/EBP α cells (Fig. 1A). The expression of other important components of insulin signaling and transporter trafficking was not very different in the two cell lines (Fig. 2D). Insulin

receptor expression was slightly lower in the NIH-PPAR γ adipocytes than in the C/EBP α cells, whereas IRS-1 levels were slightly higher in the NIH-PPAR γ adipocytes. The PI3-kinase and Akt2 levels were approximately the same, as were components of the SNARE machinery, VAMP2 and Syntaxin-4, which are necessary for Glut4/IRAP trafficking (43, 48, 59, 60).

FIG. 4. Insulin-sensitive and PI3-kinase-dependent translocation of IRAP in NIH-PPAR γ adipocytes. (A) On day 8 or 9 of differentiation, cells were serum starved for 2 h and then treated with 1 μ M wortmannin for 40 min or left untreated. The cells were treated with 100 nM insulin (+) or left untreated (–), as indicated, while still in the presence of wortmannin or carrier. The cells were then exposed to sulfo-NHS-SS-biotin (final concentration, 1 mM) for the last 2 min of the incubation. Protein (400 μ g) from whole-cell extracts was incubated with streptavidin-agarose beads overnight, and the eluted material was separated by SDS-PAGE and subjected to Western blotting with anti-IRAP antibody or anti-TfR antibody. Detection was as described in the legends to Fig. 1 and 2. (B) The extent of translocation was determined quantitatively, as described in Materials and Methods, and the mean of three independent experiments is shown. Error bars denote standard error.

FIG. 5. Formation of an intracellular, insulin-responsive compartment in NIH-PPAR γ adipocytes. On day 9 of differentiation, cells were serum starved for 2 h and then treated with 100 nM insulin (+) or for 30 min or left untreated (-). Subcellular fractions were prepared, and 750 μ g of LM fraction was centrifuged at $277,000 \times g$ for 55 min in a 4.6-ml 10 to 30% sucrose gradient. Fractions were collected from the bottom, and even membrane fractions were analyzed by Western blotting for Glut1, IRAP, and Glut4. Detection was done by using HRP-conjugated secondary antibodies and chemiluminescence. A.U., arbitrary units.

Based on these expression data, the most likely explanation for the lack of insulin-stimulated glucose uptake in the NIH-PPAR γ adipocytes is the lack of Glut4 expression, assuming that insulin signaling is normal in these cells.

Therefore, we focused our attention on an important downstream target of insulin signaling required for vesicular trafficking, namely, Akt2. Cells were stimulated with 100 nM insulin or left untreated, and whole-cell extract was then analyzed by Western blotting for phosphorylated Akt (p-Akt, phospho-Ser473). As expected, the NIH-C/EBP α adipocytes demonstrated insulin-induced phosphorylation of Akt2 (Fig. 3, right panel). The NIH-PPAR γ -expressing adipocytes, whose insulin-stimulated glucose uptake was dramatically compromised, exhibited insulin-stimulated, wortmannin-sensitive phosphorylation of Akt2 with no change in the total amount of Akt2 during the course of the experiment (Fig. 3). These data confirm that the insulin signaling to Akt2 that is required for glucose transporter translocation (3, 9, 24, 28, 35, 45) remains intact in PPAR γ -expressing adipocytes that lack C/EBP α .

Since the level of Glut4 in NIH-PPAR γ -expressing adipocytes is so low, we turned our attention to insulin-stimulated IRAP translocation since this protein resides in the Glut4-containing compartment in adipocytes and skeletal muscle and displays virtually identical localization and insulin-dependent trafficking to that of Glut4 (32, 34, 38, 44). It is therefore considered a surrogate marker that can be used to monitor insulin-stimulated translocation of vesicles to the cell surface. We used a cleavable, cell-impermeable biotin analogue (EZ-

Link-sulfo-NHS-SS-biotin) (17, 32, 33, 50) to label IRAP present at the cell surface. Figure 4A shows that IRAP translocates to the cell surface in response to insulin in a wortmannin-dependent manner in the NIH-PPAR γ -expressing adipocytes. The threefold response to insulin for translocation of IRAP for the NIH-PPAR γ adipocytes (Fig. 4B) is comparable to that seen in the NIH-C/EBP α adipocytes (data not shown) as well as in 3T3L1 cells (50, 51). This suggests that in adipocytes lacking C/EBP α , there exists significant insulin-induced translocation of Glut4 vesicle proteins and that this translocation is PI3-kinase dependent. Transferrin receptor (TfR), a marker of the general endosomal recycling machinery, also traffics to an extent in response to insulin in 3T3L1 cells (51) and shows partial colocalization with Glut4 in these cells. It is also readily biotinylated at the cell surface and translocates to the plasma membrane in response to insulin in the NIH-PPAR γ -expressing adipocytes (Fig. 4).

To monitor the translocation of Glut4 vesicle proteins from the intracellular compartment and to determine whether they could potentially be colocalized in intracellular vesicles, we performed a sucrose velocity gradient analysis, as shown in Fig. 5. Day 9 adipocytes were fractionated by the method of El-Jack et al. (13) in the presence or absence of 100 nM insulin, and a portion of the LM fraction was run on a 10 to 30% sucrose velocity gradient. An equal proportion of each fraction was subjected to SDS-PAGE followed by Western blot analysis. Our results demonstrate a loss of IRAP and Glut1 from the LM in response to insulin, indicative of their movement to the

FIG. 6. Anti-Glut4 antibody immunoadsorption adsorbs IRAP in NIH-C/EBP α adipocytes but not in the NIH-PPAR γ adipocytes. 1F8 antibody- or nonspecific IgG-coupled beads (10, 25, or 50 μ l) were incubated with 200 μ g of LM from NIH-PPAR γ and NIH-C/EBP α adipocytes (day 9) overnight, washed, and eluted sequentially with 1% Triton X-100 in PBS followed by SDS-containing Laemmli sample buffer (nonreducing), as described in Materials and Methods. Equal proportions of supernatant (unbound), Triton X-100 eluate (colocalized proteins), and SDS eluate (bound Glut4) were electrophoresed in 10% acrylamide gels and immunoblotted for the indicated proteins. Detection was done by using HRP-conjugated secondary antibodies and chemiluminescence.

cell surface (Fig. 4) and consistent with data obtained with 3T3L1 adipocytes. These data suggest that in NIH-PPAR γ adipocytes, there exists an insulin-sensitive compartment containing IRAP and Glut1. Although Glut4 expression was very low in these cells, a sufficient exposure of the gels demonstrated its cosedimentation with IRAP and Glut1 (but see below).

In Fig. 6, we show the results of an immunoadsorption experiment in which LM from NIH-PPAR γ and NIH-C/EBP α -expressing adipocytes were incubated with anti-Glut4 antibody coupled to acrylic beads. At the highest antibody level, corresponding to 50 μ l of beads, 75% of Glut4 was adsorbed by the beads. For the NIH-C/EBP α adipocytes, approximately one-third of the IRAP was pulled down by the beads, indicating that there is considerable colocalization between the two proteins in the LM fraction. However, in the NIH-PPAR γ -expressing adipocytes, most of the Glut4 was adsorbed by the beads but there was no colocalization with IRAP. The lack of colocalization between Glut4 and IRAP in the NIH-PPAR γ adipocytes is the result of the very low levels of Glut4 expression relative to IRAP, and so the majority of IRAP cannot be immunisolated in this manner, despite its existence in an intracellular insulin-responsive pool.

Therefore, the NIH-PPAR γ -expressing adipocytes produce a large pool of insulin-sensitive IRAP-rich vesicles that have similar characteristics to the IRVs/GSVs present in 3T3L1 and primary rat adipocytes, except that they have minimal Glut4. We would predict that increasing the Glut4 levels by transfection or infection would result in Glut4 being targeted to these IRVs. Therefore, we expressed myc-tagged Glut4 (Glut4-myc) via retroviral expression in the NIH-PPAR γ cells, as shown in

Fig. 7. As detected with 1F8 (monoclonal anti-Glut4 antibody) or with 9B11 (monoclonal anti-Myc-Tag antibody), Glut4-myc expression was induced earlier in the differentiation process to a level about twice as high as that of endogenous Glut4. The

FIG. 7. Restoration of Glut4 expression in NIH-PPAR γ adipocytes. On the indicated days after induction, whole-cell extracts were prepared from differentiating NIH-PPAR γ cells expressing Glut4-myc (see Materials and Methods) or from day 9 NIH-PPAR γ adipocytes (parental). Equal amounts of protein (250 μ g) were resolved by SDS-PAGE and then immunoblotted for Glut4 (1F8), Glut4-myc (9B11 anti-Myc Tag antibody or 1F8), Glut1, and IRAP. Detection was as described in the legends to previous figures.

FIG. 8. Restoration of Glut4 expression in NIH-PPAR γ adipocytes results in insulin-stimulated translocation of Glut4 to the plasma membrane. Cells were treated with 100 nM insulin (b, c, e, and f) or carrier (a and d) and then fixed and treated as described in Materials and Methods. Anti-Myc-Tag antibody (9B11) was used to detect the translocation of Glut4 to the plasma membrane. Panels d to f are light microscopic images of the exact same plane in which the fluorescent images (panels a to c) are from and at the indicated magnifications.

levels of Glut1 and IRAP remained unchanged compared to the parental NIH-PPAR γ cells when Glut4-myc was ectopically expressed (Fig. 7).

The ectopic Glut4-myc protein translocated to the plasma membrane in response to insulin, as shown by immunofluorescence using an anti-Myc-Tag antibody (Fig. 8). The cells were not permeabilized, and thus only the Glut4 that had fused with the plasma membrane was detected; it was invisible in the basal state. This result further confirms that an insulin-responsive vesicular pool was formed in adipocytes lacking C/EBP α and that it was present in the NIH-PPAR γ adipocytes, despite the lack of Glut4 expression.

To determine the extent of insulin-dependent translocation to the plasma membrane for ectopically expressed Glut4-myc, cells were exposed to insulin or left untreated and were then incubated with anti-Myc-Tag antibody or mouse IgG as described in Materials and Methods. As shown in Fig. 9, there was some ectopically expressed Glut4-myc at the plasma membrane in the basal state and there was a threefold increase in the cell surface Glut4 myc level as a result of insulin-mediated translocation to the cell surface. This threefold increase was essentially identical to that observed for IRAP translocation in response to insulin, as assessed by cell surface biotinylation (Fig. 4). If the cells were permeabilized during the antibody incubation so that all the Glut4-myc was accessible to labeling,

then insulin had no effect on the amount of antibody that was labeled and subsequently immunoprecipitated (data not shown). We interpret these results to indicate that the ectopically expressed Glut4-myc is directed to a preexisting pool of insulin-responsive vesicles, which translocate to the plasma membrane on insulin signaling. However, the twofold increase in the total Glut4 levels due to Glut4-myc expression in the NIH-PPAR γ + Glut4-myc adipocytes was insufficient to observe a significant increase in insulin-stimulated 2-deoxyglucose uptake (data not shown) since the transporter was still expressed at levels below functional significance.

DISCUSSION

To efficiently clear glucose from the blood after feeding, the insulin-sensitive tissues of fat and muscle elaborate an abundant population of intracellular vesicles that are enriched in two major cargo proteins, Glut4 and IRAP. These proteins move to the cell surface in response to insulin, and the former serves to transport glucose into the tissues whereas the exact physiological role of the latter in the context of insulin action remains uncertain. The targeting and trafficking of Glut4 and IRAP is complex and incompletely understood (53). Simple overexpression of Glut4 in a variety of cell types does not produce insulin-regulated glucose transport like that of mature

FIG. 9. Quantitative evaluation of Myc-tagged Glut4 translocation to the plasma membrane in response to insulin. (A) Differentiated cells (day 10) were treated with 100 nM insulin (+) or carrier (–) for 30 min, as in previous figures. The cells were cooled to 4°C in PBS and incubated with anti-Myc-Tag (9B11) antibody (or IgG). Following the incubation, the cells were washed three times with ice-cold PBS to remove any excess antibody and then lysed in a 1% Triton X-100 buffer containing protease inhibitors as described in Materials and Methods. Whole-cell extracts from each of the samples were prepared, and 200 μ g was incubated for 3 h with protein A-agarose beads. Immunoprecipitated proteins were eluted from the beads and resolved by SDS-PAGE analysis followed by Western blotting with either anti-Myc-Tag antibody or anti-GLUT4 antibody. Detection was done by using HRP-conjugated secondary antibodies and chemiluminescence. (B) The extent of translocation was determined quantitatively, as described in Materials and Methods, and the mean of three independent experiments is shown. Error bars indicate standard error. A.U., arbitrary units.

fat and muscle (21, 25, 52, 62), although a compartment that resembles IRVs/GSVs can be detected in fibroblastic cells at levels that are well below those seen in classical insulin target tissue (2, 7, 22, 29, 40). In this study we examined the roles of the adipogenic transcription factors, C/EBP α and PPAR γ , in the formation of IRVs/GSVs and the expression of their major cargo proteins. We found that in the context of the NIH 3T3 cell background, both C/EBP α and PPAR γ expression cause their conversion to adipocytes, as we have previously shown (12). Cells expressing the latter exhibit minimal Glut4 expression and no insulin-stimulated glucose transport, whereas the former cells resemble 3T3L1 cells in having a significant extent of insulin-stimulated glucose transport. However, the PPAR γ expressing cells have abundant IRAP, which shows a significant insulin-dependent translocation to the cell surface. We conclude that a major role of C/EBP α is to regulate Glut4 expression and that the IRVs/GSVs can form in the virtual absence of this protein.

Some indirect information about the possible role of IRAP and Glut4 in the formation of IRVs/GSVs has been obtained from studies of cells derived from knockout and transgenic animals. Global elimination of Glut4 results in increased IRAP expression in adipocytes and decreased expression in skeletal muscle (36), with little, if any, insulin responsiveness as as-

sessed by subcellular distribution (27). On the other hand, the LM pool of IRAP in the adipocytes from these animals was quite large but was not further characterized, by gradient analysis for example. Therefore, it is not clear if cells from these animals have an IRV/GSV compartment equivalent to that of the wild-type animal. Glut4 expression is decreased in all insulin-responsive tissues from IRAP knockout animals, yet the animals are not insulin resistant or diabetic (37). This suggests that an IRV/GSV compartment can exist without IRAP, but the intracellular Glut4 compartment(s) from these animals was not studied.

Regarding the contributions of PPAR γ and C/EBP α to the development of the mature adipocyte phenotype, we and others have previously shown that fat cells lacking C/EBP α expression are unable to take up glucose in response to insulin (12, 65). In the latter study, Glut4 levels were relatively high but insulin receptor and IRS-1 expression and their tyrosine phosphorylation were decreased, suggesting that this may be the cause of their lack of insulin-responsive glucose uptake (65). On the other hand, our previous study attributed the lack of response to the undetectable levels of Glut4 expression (12), a result which we confirm here, keeping in mind that the engineered adipocytes in these two studies were derived from different sources. Therefore, we now show that insulin receptor signaling to vesicular trafficking (Fig. 4 and 5) is normal in the PPAR γ -expressing adipocytes that lack C/EBP α . What, then, is the reason for these discrepancies? One possibility is that the cells derived from the C/EBP α knockout animals may have compensatory overexpression of other C/EBPs that can act on the C/EBP α -binding site that has been shown to be present in the Glut4 gene and is necessary, at least in part, for Glut4 expression (15, 23, 30, 41, 57). Alternatively, another compensatory gene expression may explain the difference. However, in the PPAR γ -expressing adipocytes used in the present study, there is no detectable C/EBP α (Fig. 1B) and C/EBP β/δ expression is the same as that seen in 3T3L1 cells (data not shown).

The question remains of how closely the IRAP-rich compartment we describe here resembles the IRV/GSVs of 3T3L1 cells, their lack of Glut4 notwithstanding. The level of IRAP expression is essentially the same in these cell lines (Fig. 2C), and the sedimentation pattern of intracellular vesicles rich in IRAP is the also same (compare Fig. 5 above and Fig. 6 from reference 13). IRAP translocates to the cell surface in an insulin-sensitive manner (Fig. 4 and 5). Moreover, there is virtually complete intracellular sequestration of transfected Myc-tagged Glut4 in the basal state, and it exhibits insulin-dependent movement to the cell surface (Fig. 8 and 9), just as in other fat cell lines (2, 31). These data, taken together, strongly suggest that GSVs/IRVs form during the course of differentiation, despite the absence of Glut4.

These data suggest that the mechanism which sequesters these vesicles does not depend solely on the presence of Glut4 or, more specifically, regions in the cytoplasmic portion of this transporter, including the C terminus. It has been shown that microinjection of IRAP peptide (positions 1 to 109) results in translocation of Glut4 to the plasma membrane in the absence of insulin (64) and that the slow recycling of an IRAP chimera (containing the cytosolic/N-terminal domain of IRAP fused to TfR) depends on an intact cytosolic IRAP domain containing the dileucine motif (29). Therefore, these cells, expressing min-

imal amounts of Glut4, represent a useful tool to study the involvement of IRAP in the retention and sorting mechanism of GSVs and its potential association with the targeting and tethering proteins involved in this process. Such studies are under way.

Thus, based on previous data (13, 68) and data presented here, we would propose that Glut4 is not necessary for the formation of an insulin-responsive vesicle in adipocytes and that it is simply targeted to a preexisting vesicle which has formed independently of Glut4. We would also conclude that C/EBP α expression is not a requirement for insulin sensitivity in adipose cells. There is certainly a subset of genes, which are turned on by C/EBP α during adipocyte differentiation, which are important for the formation of a mature fat cell. However, insulin sensitivity, as measured by insulin receptor signaling and vesicle translocation, is maintained in the absence of C/EBP α .

ADDENDUM

Since the submission of this paper, a study by Carvalho et al. (8a) demonstrated that in adipocytes from mice having ca. 10% of the wild-type Glut4 expression, there was decreased expression of IRAP and VAMP2 proteins, but these nonetheless showed insulin-dependent translocation to the cell surface. These data also suggest the existence of "Glut4 storage vesicles" in the absence of functionally significant Glut4.

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