The Transcription Factor CCAAT/Enhancer-binding Protein α Is Required for the Intracellular Retention of GLUT4*

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Insulin modulates glucose uptake into adipocytes by regulating the trafficking of the GLUT4 glucose transporter. GLUT4 is mostly excluded from the surface of unstimulated cells because it is much more slowly exocytosed than it is endocytosed. GLUT4 traffics through an adipocyte-specific, specialized endosomal recycling pathway that only partially overlaps with compartments of the general endosomal recycling pathway. Insulin stimulates GLUT4 exocytosis and partially inhibits its endocytosis, resulting in GLUT4 redistribution to the cell surface. Insulin does not stimulate glucose uptake into adipocytes lacking the CCAAT/Enhancer-binding protein α (C/EBPα) transcription factor. Here we show that these adipocytes do not properly traffic GLUT4. In these adipocytes, GLUT4 was rapidly exocytosed in basal conditions, resulting in an accumulation of GLUT4 on the plasma membrane. Although the kinetics of GLUT4 trafficking were altered, GLUT4 was still targeted to specialized intracellular compartments in adipocytes lacking C/EBPα, demonstrating an uncoupling of the targeting of GLUT4 to a specialized, adipocyte-specific insulin-regulated pathway from the regulation of the movement of GLUT4 through this pathway. Re-expression of C/EBPα in adipocytes lacking C/EBPα restored normal GLUT4 trafficking. We propose that C/EBPα controls the expression of the proteins that determine the basal, slow exocytosis of GLUT4, but not the proteins required to make the adipocyte-specific compartments through which GLUT4 traffics. Furthermore, these data support a model in which insulin stimulates GLUT4 exocytosis by releasing an inhibitor of GLUT4 movement to the cell surface, and it is this clamp on basal exocytosis that is missing in adipocytes lacking C/EBPα.

Insulin regulates whole body glucose homeostasis, in part, by regulating the uptake of glucose into fat and muscle cells. Insulin regulates glucose uptake by modulating the distribution of the GLUT4 glucose transporter between the surface and interior of cells (1, 2). GLUT4 is mostly retained intracellularly in basal conditions, and insulin stimulates a redistribution of GLUT4 to the cell surface, where it functions to allow glucose to enter cells by diffusing down the concentration gradient. In basal conditions, GLUT4 is much more rapidly internalized than exocytosed, resulting in its intracellular retention by a dynamic mechanism (3, 4, 29). Insulin stimulates exocytosis and inhibits internalization, and both of these changes promote a net redistribution of GLUT4 to the cell surface.

Insulin-regulated membrane trafficking is highly specialized. GLUT4, the only glucose transporter family member whose distribution is markedly modified by insulin, is almost exclusively expressed in fat and muscle, the two tissues with pronounced insulin-regulated glucose uptake. IRAP, a transmembrane insulin-regulated aminopeptidase, is the only other protein known to traffic through the GLUT4 pathway (5, 6). The physiological role of IRAP is not known (7). In addition, a facilitated recycling pathway being highly selective for cargo molecules (e.g. GLUT4 and IRAP), this specialized trafficking pathway is most highly developed in fat and muscle cells, the two cell types involved in disposal of dietary glucose.

The complex intracellular itinerary of GLUT4 has been intensively studied, and although the pathway has not been completely elucidated, it is clear that GLUT4 traffics through both general endosomes and specialized compartments as it cycles between the interior and cell surface (8, 9). GLUT4 enters cells through clathrin-mediated endocytosis and is delivered to endosomes along with other proteins internalized by the clathrin pathway (10). Most membrane proteins are rapidly recycled back to the cell surface from endosomes. GLUT4 does not return directly from endosomes to the cell surface, but rather it is transported to an intracellular compartment that is not along the general endocytic-recycling pathway. Here we refer to this compartment as specialized to distinguish it from general endosomes (3, 8). In unstimulated 3T3-L1 adipocytes, intracellular GLUT4 is nearly equally distributed between endosomes and the specialized compartment, indicating that in unstimulated cells, GLUT4 is stored in both endosomes and the specialized compartment (8, 9).

GLUT4 is much more slowly exocytosed than membrane proteins that cycle through the general endocytic recycling pathway (3, 4, 11, 12). Transport of GLUT4 or IRAP to the specialized compartment correlates with their slow, insulin-regulated efflux. In 3T3-L1 adipocytes, GLUT4 and IRAP are segregated from endosomes and slowly recycled, whereas in 3T3-L1 preadipocytes or fibroblast-like cell types, ectopically expressed GLUT4 is not segregated from endosomes and is not as slowly exocytosed as in differentiated cells (8, 13, 14). In muscle cells, the other cell type with insulin-regulated glucose uptake, GLUT4 is also partially segregated from endosomes (15–17). The details of how the specialized pathway is formed in differentiated cells and how traffic through this pathway is regulated by insulin are not known.

The transcription factors PPARγ1 and C/EBPα are critical regulators of adipogenesis (18–20). PPARγ is the more prox-

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1 The abbreviations used are: PPARγ, peroxisome proliferator-activated receptor γ; C/EBP, CCAAT/Enhancer-binding protein α; HA, hemagglutinin; HRP, horseradish peroxidase; Tf, transferrin; DAB, diaminobenzidine; TR, transferrin receptor.
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mal regulator of adipogenesis, since immortalized mouse embryonic fibroblasts lacking PPARγ cannot be differentiated into adipocytes, whereas immortalized embryonic fibroblasts lacking C/EBPα can be differentiated into adipocytes when PPARγ is ectopically expressed (19, 21). Adipocytes lacking C/EBPα accumulate lipid and express many adipocyte proteins, including GLUT4 (21, 22). However, adipocytes lacking C/EBPα are not completely normal, since insulin does not stimulate glucose uptake into these cells (21, 22). The C/EBPα−/− adipocytes exhibit reduced expression of insulin receptor and insulin receptor substrate-1, which may partly explain why these cells are insulin-insensitive (21).

Here we report on the characterization of GLUT4 trafficking in adipocytes derived from C/EBPα−/− mouse embryonic fibroblasts. The objectives of these studies were to understand why adipocytes lacking C/EBPα have elevated basal glucose uptake and are not responsive to insulin (21, 22). We found that adipocytes lacking C/EBPα segregated GLUT4 from endosomes to the same degree as in wild type adipocytes, but unlike wild type adipocytes, GLUT4 is rapidly exocytosed in the basal state. The trafficking of IRAP is also altered in adipocytes lacking C/EBPα, indicating that the insulin-regulated trafficking pathway is perturbed rather than the effects being restricted to GLUT4 behavior. These data demonstrate that C/EBPα controls the expression of proteins that regulate the kinetics of GLUT4 exocytosis, but not the expression of proteins required to make the specialized GLUT4-containing compartment in adipocytes. Thus, in these mutant adipocytes, the formation of the specialized insulin-regulated pathway, characteristic of adipocytes, is uncoupled from the regulation of traffic through this pathway. The failure of insulin to stimulate glucose uptake above basal levels in adipocytes lacking C/EBPα is explained by the accumulation of GLUT4 on the surface in the basal state, establishing that the insulin resistance of these cells is, in part, due to a defect in the basal trafficking kinetics of GLUT4.

MATERIALS AND METHODS

Ligands and Chemicals—Unless noted otherwise, all chemicals were purchased from Sigma. Fluorescent antibodies were purchased from Jackson Immunolabs, Inc. (West Grove, PA). Mouse anti-HA monoclonal antibody (HA.11) was purified from ascites (Covance, Berkley, CA) on a protein G affinity column (Amersham Biosciences). The concentration of the antibody required to saturate the HA epitope of HA-GLUT4-GFP was measured as described previously (8). Human transferrin (Sigma) was conjugated to horseradish peroxidase as described previously (3). The C/EBPα and PPARγ retroviral expression vectors have been described (23). Anti-Akt and anti-phosphoserine 473 antibodies were purchased from Cell Signaling Inc (Beverly, MA).

Cell Culture—Mouse embryonic fibroblast cells for this work were kindly provided by Gretchen Darlington, Evan Rosen, and Bruce Spiegelman (21, 24). C/EBPα-deficient cells were infected with PPARγ retroviral vector or PPARγ and C/EBPα retroviral vectors as described previously (21, 23). Cells were cultured in Dulbecco’s modified Eagle’s medium with 10% calf serum and selected for PPARγ with 2 μg/ml puromycin or for C/EBPα with 5 μg/ml hygromycin for a week after infection. Pooled selected cells were used for up to eight passages and then discarded. Selected cells were differentiated and electroporated as previously described (8).

Trafficking Assays—The surface-to-total ratios of HA-GLUT4-GFP, IRAP-transferrin receptor (IRAP-TR), and TR, and the eocytic rate constant measurements for HA-GLUT4-GFP were measured as previously described (3, 8, 25, 26).

Distribution between Endosomes and the Specialized Compartment—The distribution of HA-GLUT4-GFP between endosomes and the specialized compartment was calculated as previously described (3, 8, 25, 26). Briefly, cells electroporated with HA-GLUT4-GFP and TR or IRAP-TR were incubated for 6 h with a saturating concentration of HA.11 anti-HA antibody (typically 50 μg/ml) with or without 20 μg/ml HRP-Tf. During this incubation, the complete cellular pool of HA-GLUT4-GFP was bound by HA.11 as the GLUT4 construct cycled between the surface and the interior of cells. After this incubation, the cells were placed on ice, and surface-bound HRP-Tf was removed by washing the cells twice with ice-cold citrate buffer (20 mM sodium citrate and 150 mM NaCl, pH 5.0), followed by a wash with neutral pH buffer. The cells were incubated for 30 min with 250 μg/ml diamonobenzidine (DAB) and 0.0025% H2O2 in the dark. The cells were washed twice and fixed with 3.7% formaldehyde. HA.11 on the surface (bound to HA-GLUT4-GFP) was bound with a saturating concentration of unlabeled donkey anti-mouse IgG antibody. The cells were refluxed, and the amount of intracellular HA-11 was determined by indirect immunofluorescence of saponin-permeabilized cells using donkey anti-mouse IgG directly labeled with Cy3. The Cy3 fluorescence in each cell was normalized to the GFP fluorescence in the cell. The Cy3/GFP ratio normalizes the nonablated Cy3 fluorescence to the total amount of HA-GLUT4-GFP expressed per cell. Fluorescence was also collected from cells that were not incubated with HRP-Tf but were treated with DAB and H2O2. This fluorescence is the total nonablated Cy3/GFP ratio. To calculate the fraction of HA-GLUT4-GFP in the specialized compartment the following was used: fTR = fHA-11/TR/TOTAL − fHA-11/HRP-TR, where fHA-11 is the Cy3/GFP fluorescence remaining after ablation by TR-HRP taken up via the TR, fHA-11/TR is the fluorescence remaining after ablation by TR-HRP taken up via the IRAP-TR, and fTOTAL is the fluorescence in cells that were not incubated with TR-HRP (no ablation).

Epitope ablation in undifferentiated cells was measured by growing the cells to 80% confluence on 6-well plates coated with poly-γ-lysine. Cells were transfected with 3.7 μg of HA-GLUT4-GFP′ together with either 5.5 μg of TR or IRAP-TR, using LipofectAMINE 2000. The following day, the cells were plated on coverslip dishes, and the epitope ablation assay was performed as described above.

Fluorescence Quantification—The images were collected on a DMI880 inverted microscope (Leica Inc., Deerfield, IL) with a cooled CCD camera (Princeton Instruments Inc., West Chester, PA). All images were taken using a 40 × 1.25 numerical aperture oil immersion objective. The measurements and quantifications were performed as described previously (3, 8, 14).

RESULTS

C/EBPα−/− Adipocytes Do Not Retain GLUT4 Intracellularly in the Basal State—To better understand why insulin does not regulate glucose uptake into C/EBPα−/− adipocytes, we characterized the trafficking behavior of GLUT4 in these cells. C/EBPα−/− mouse embryonic fibroblasts, expressing PPARγ by retroviral infection, were differentiated into adipocytes (21). Here we refer to adipocytes derived from C/EBPα−/− fibroblasts expressing exogenous PPARγ by retroviral infection as C/EBPα−/− adipocytes. On day 4 of differentiation, the cells were electroporated with HA-GLUT4-GFP, a GLUT4 construct with an HA epitope inserted in the first exofacial loop and GFP fused to the carboxyl terminus of GLUT4 (27, 28). The distributions of HA-GLUT4-GFP in basal and insulin-stimulated conditions were determined on the following day (8). HA-GLUT4-GFP on the surface was measured by indirect immunofluorescence of fixed cells using an anti-HA antibody (HA.11), and total expression of the reporter was measured by the GFP fluorescence per cell. HA-GLUT4-GFP, detected by GFP fluorescence, was concentrated in the perinuclear region and in small punctate structures distributed throughout unstained C/EBPα−/− adipocytes, a pattern indistinguishable from that in 3T3-L1 adipocytes (Fig. 1). However, in C/EBPα−/− adipocytes, there was a significant amount of surface HA.11 staining, whereas in 3T3-L1 adipocytes, there was little HA.11 surface staining in basal conditions. Thus, basal C/EBPα−/− adipocytes do not properly retain GLUT4 intracellularly. The expression of HA-GLUT4-GFP on the plasma membranes of the two cell types stimulated with insulin was qualitatively indistinguishable (Fig. 1).

Quantification of the HA-GLUT4-GFP surface-to-total distributions revealed a robust translocation to the surface of 3T3-L1 adipocytes and no significant redistribution in the C/EBPα−/− adipocytes (Fig. 2A). The major difference between 3T3-L1 and C/EBPα−/− adipocytes was in the basal state, with the surface-to-total GLUT4 in adipocytes lacking C/EBPα significantly increased relative to 3T3-L1 adipocytes, demonstrating that
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C/EBPα−/− Adipocytes Do Not Retain IRAP—We next determined whether the distribution of IRAP, a protein known to traffic by the insulin-regulated pathway in adipocytes, was altered in the C/EBPα knockout adipocytes. We used a chimera containing the cytoplasmic domain of IRAP fused to the transmembrane and extracellular domains of the human TR. We have previously shown that this chimera, IRAP-TR, traffics by the insulin-regulated pathway in 3T3-L1 adipocytes (8). In the C/EBPα−/− adipocytes, as compared with 3T3-L1 adipocytes, IRAP-TR was not retained intracellularly in the basal state, nor did insulin induce a large redistribution of IRAP-TR to the cell surface (Fig. 2B). Thus, like GLUT4, IRAP is not retained intracellularly in unstimulated C/EBPα−/− adipocytes. This demonstrates that in the absence of C/EBPα, the basal trafficking parameters of the insulin-regulated pathway are affected, rather than the altered trafficking being restricted to GLUT4.

Transferrin Receptor Distribution Is Normal in C/EBPα−/− Adipocytes—In unstimulated adipocytes, GLUT4 and IRAP are dynamically retained intracellularly by rapid internalization from the plasma membrane and slow return to the cell surface (e.g. see Refs. 3, 4, 12, and 29). To investigate whether the effect of C/EBPα knockout is restricted to the specialized trafficking of GLUT4 and IRAP, we examined the behavior of the TR, a commonly used marker of the general endocytic recycling pathway. The surface-to-total distribution of the TR in the C/EBPα−/− adipocytes was similar to that in 3T3-L1 adipocytes in both the unstimulated and stimulated conditions (Fig. 2C). These data indicate that the altered surface-to-total distributions of GLUT4 and IRAP in adipocytes lacking C/EBPα did not result from gross changes in general membrane trafficking, since such changes would probably have altered TR behavior. Because unstimulated C/EBPα−/− adipocytes did not retain GLUT4 and IRAP, insulin had no greater effect on surface expression of GLUT4 and IRAP than it did on the TR (Fig. 2D). The analysis of the HA-GLUT4-GFP surface-to-total distribution indicates that the previously documented failure of insulin to stimulate glucose uptake above basal levels in C/EBPα−/− adipocytes is the result of a defect in insulin-regulated trafficking.

Exogenously Expressed C/EBPα Restores GLUT4 Retention and Insulin-stimulated Redistribution to C/EBPα−/− Adipocytes—We next determined whether exogenously expressed C/EBPα would restore intracellular retention of GLUT4 in the C/EBPα−/− adipocytes. C/EBPα−/− embryonic fibroblasts were infected with retroviruses containing C/EBPα and PPARγ. HA-GLUT4-GFP expressed in adipocytes derived from these doubly infected C/EBPα−/− fibroblasts was excluded from plasma membrane in basal conditions, and it was redistributed to the cell surface by insulin (Fig. 3A). Reintroduction of C/EBPα resulted in an about 40% decrease in HA-GLUT4-GFP on the surface of unstimulated cells (Fig. 3B). Coincident with the restored intracellular retention of GLUT4, insulin stimulated a 2–3-fold translocation of HA-GLUT4-GFP to the cell surface (Fig. 3C). The magnitude of translocation of GLUT4 in C/EBPα−/− adipocytes re-expressing C/EBPα was similar to that in adipocytes derived from C/EBPα−/− mouse embryonic fibroblasts (Fig. 3C). Furthermore, the magnitude of the translocation was similar to the insulin-stimulated increase in glucose uptake previously measured in C/EBPα−/− adipocytes ectopically expressing C/EBPα and in adipocytes derived from NIH-3T3 expressing C/EBPα (21, 30). Taken together, these data indicate that re-expression of C/EBPα restored insulin-regulated trafficking of GLUT4 and insulin-stimulated glucose uptake.

Although re-expression of C/EBPα restored basal retention and insulin-stimulated translocation of GLUT4, neither reta-
Distributions of HA-GLUT4-GFP, IRAP-TR, and TR in C/EBPα−/− and 3T3-L1 adipocytes. A, quantification of the surface/total ratios of HA-GLUT4-GFP. The surface/total ratios were normalized to the ratio in basal 3T3-L1 adipocytes. Surface HA-GLUT4-GFP was measured in indirect immunofluorescence of the HA epitope with HA-11 antibody and a Cy3-labeled secondary antibody. GFP fluorescence was used to normalize the Cy3 data for the total HA-GLUT4-GFP expressed per cell. The data are the averages calculated in 15 independent, matched experiments (mean ± S.E.). B, quantification of surface/total ratios of IRAP-TR. The surface/total ratios were normalized to the ratio in basal 3T3-L1 adipocytes. Surface IRAP-TR was measured in indirect immunofluorescence with B3/25 antibody, which binds the extracellular domain of the human transferrin receptor sequences of IRAP-TR, followed by an Alexa488-labeled secondary antibody. Cy3-labeled transferrin taken up from the medium during a 6-h incubation was used to measure the total amount of IRAP-TR expressed per cell. The data are the average ± S.D. of two experiments. C, quantification of surface/total ratios of TR. The surface/total ratios were normalized to the basal ratio in 3T3-L1 adipocytes. The TR surface/total ratio was measured using the method as described in B. The data are the average ± S.E. of four independent matched experiments. In each case, the surface/total ratios reflect the relative amount of the reporters on the cell surface in the two different cell types. D, the magnitude of the insulin-stimulated redistributions of the three different reporters in the two cell types are plotted as the ratio of the surface/total distributions in insulin-stimulated to unstimulated cells.

In the adipocytes lacking C/EBPα, we found, using anti-phosphoserine 473 antibody in Western blotting, that little Akt was phosphorylated on serine 473 in basal conditions and that insulin stimulated phosphorylation of serine 473. This effect was similar to insulin-stimulated phosphorylation of Akt in C/EBPα+/− adipocytes re-expressing C/EBPα as well as in 3T3-L1 adipocytes (not shown). Thus, the phenotype of GLUT4 trafficking in C/EBPα−/− adipocytes is not because Akt is hyperactivated in these cells, arguing against persistent insulin signaling as the underlying cause of the altered surface-to-total distribution of GLUT4 in C/EBPα−/− adipocytes.

GLUT4 Is Rapidly Exocytosed in C/EBPα−/− Adipocytes—GLUT4 is dynamically retained in unstimulated adipocytes because GLUT4 is more rapidly endocytosed than exocytosed. The failure of C/EBPα−/− adipocytes to properly retain GLUT4 could result from alterations in endocytosis, in exocytosis, or in both parameters. GLUT4, like the TR, is internalized through clathrin-coated pits (10); therefore, if GLUT4 redistribution to the surface of C/EBPα−/− adipocytes was due to an inhibition of clathrin-coated pit internalization, then the TR would also be increased on the surface of these cells compared with adipocytes expressing C/EBPα. The distribution of the TR between adipocytes expressing exogenous C/EBPα+/− and C/EBPα−/− in 3T3-L1 adipocytes (not shown). Thus, the phenotype of GLUT4 trafficking in C/EBPα−/− adipocytes is not because Akt is hyperactivated in these cells, arguing against persistent insulin signaling as the underlying cause of the altered surface-to-total distribution of GLUT4 in C/EBPα−/− adipocytes.
the HA-GLUT4-GFP in the C/EBP
C
Total HA-GLUT4-GFP expressed per cell was determined by GFP fluorescence. Face HA-GLUT4-GFP was calculated from indirect fluorescence measurement using anti-HA primary antibody and a Cy3 secondary antibody.

clathrin-mediated endocytosis is not altered in adipocytes lacking C/EBP
PPAR
knockout. C/EBP
HA-GLUT4-GFP distributions were determined as in Fig. 1. C/EBP
adipocytes and H11011
H9251
surface-to-total ratio in unstimulated cells, normalized to the ratio in C/EBP
adipocytes expressing exogenous C/EBP
and C/EBP
retrovirus. The translocation values for C/EBP
adipocytes expressing exogenous C/EBP
are from five experiments ± S.E., and the data for C/EBP
adipocytes are from two experiments ± S.D.

the interior and surface of C/EBPα−/− adipocytes, which was similar to that in 3T3-L1 adipocytes (Fig. 2C), was not altered by re-expression of C/EBPα: ~36% TR on surface of C/EBPα−/− adipocytes and ~39% TR on the surface of C/EBPα−/− adipocytes in which C/EBPα is re-expressed. These data suggest that clathrin-mediated endocytosis is not altered in adipocytes lacking C/EBPα.

To directly examine the exocytosis of HA-GLUT4-GFP, adipocytes were incubated at 37°C in medium containing HA.11. In this assay, cell-associated HA.11 increases with incubation time as unoccupied intracellular GLUT4 traffics to the cell surface, where it is bound by the HA.11 (3). HA.11 remains bound as the HA-GLUT4-GFP is internalized, traffics through intracellular compartments, and is returned to the plasma membrane. Accumulation of HA.11 reaches a plateau when the total cycling pool of HA-GLUT4-GFP is occupied with the antibody. The exocytic rate constant of HA-GLUT4-GFP is calculated from the rise in total cell-associated HA.11 to the plateau level. The re-expression of C/EBPα in C/EBPα−/− adipocytes results in a 2-fold reduction in the basal exocytosis rate constant of HA-GLUT4-GFP, establishing that C/EBPα expression has a role in regulating basal GLUT4 exocytosis kinetics (Fig. 4). The steady state amount of GLUT4 on the surface is a balance of endocytosis and exocytosis, and the slower basal exocytosis of HA-GLUT4-GFP upon re-expression of C/EBPα accounts for the reduced amount of HA-GLUT4-GFP on the surface of adipocytes expressing C/EBPα−/− adipocytes compared with C/EBPα−/− adipocytes. As noted above, re-expression of C/EBPα does not affect the distribution of the TR, providing additional evidence that the effects of C/EBPα expression are specific for GLUT4 exocytosis.

GLUT4 Is Trafficked to a Specialized Intracellular Compartment in C/EBPα−/− Adipocytes—In unstimulated adipocytes, there are two pools of intracellular GLUT4. Approximately half of GLUT4 is found in endosomes that are accessible to the TR, whereas the other half is in intracellular compartments that are not accessible to TR (3, 8, 9, 32). Both intracellular pools of GLUT4 are accessible to IRAP-TR internalized from the plasma membrane (3, 8). Because the latter intracellular pool of GLUT4 is segregated from the TR, a marker of general endocytic recycling, we refer to this as the specialized GLUT4 compartment. The mechanism by which GLUT4 exocytosis is regulated has not been completely described; however, current models propose that GLUT4 exocytosis is regulated by re-expression of C/EBPα.
slowly from the specialized compartment back to the cell surface (Fig. 5A).

The specialized GLUT4 pathway develops with differentiation, and trafficking of GLUT4 to the specialized pathway correlates with the increased GLUT4 retention characteristic of adipocytes. When GLUT4 is expressed in undifferentiated preadipocyte 3T3-L1 cells, all of the intracellular GLUT4 is localized to TR-containing endosomes (8, 13, 14). Upon differentiation, GLUT4 is partially segregated from the TR, and its intracellular retention is increased because GLUT4 exocytosis is considerably slower in adipocytes. Therefore, one possible explanation for the phenotype of the C/EBPα−/− adipocytes is that development of the specialized compartment/pathway depends on genes regulated by C/EBPα. In the absence of the specialized pathway, GLUT4 and IRAP may be rapidly exocytosed to the cell surface from the TR-containing endosomes (Fig. 5A). To determine whether the C/EBPα−/− adipocytes have the specialized GLUT4 pathway, we determined the overlap between GLUT4 and TR using a TF-HRP epitope ablation method (8, 9, 14, 32). In this procedure, adipocytes co-expressing HA-GLUT4-GFP and either IRAP-TR or TR are incubated in medium containing HA.11 and TF-HRP for 6 h. Cells are then incubated with DAB and hydrogen peroxide on ice. During this incubation, HRP catalyzes the polymerization of DAB within the lumen of compartments containing the TF-HRP. The DAB reaction product ablates epitopes within these compartments. The amount of HA.11 (bound to the luminal domain of HA-GLUT4-GFP) ablated is determined by quantitative, indirect immunofluorescence.

As we have previously shown, in 3T3-L1 adipocytes, ~80% of the total intracellular HA-GLUT4-GFP is ablated when TF-HRP is delivered to intracellular compartments by IRAP-TR, whereas only ~40% is ablated when TF-HRP is delivered by TR (Fig. 5B). Since the maximum ablation achievable by this method is 80%, we conclude that IRAP-TR has access to all of the intracellular compartments that contain HA-GLUT4-GFP (3, 8, 14, 33). The less efficient ablation of HA-GLUT4-GFP when TF-HRP was internalized by the TR demonstrates that a fraction of intracellular HA-GLUT4-GFP is in compartments that are not accessible to TR. We refer to this pool of GLUT4 as being in a “specialized” compartment to distinguish it from the GLUT4 in TR-containing endosomes. In C/EBPα−/− adipocytes, HA-GLUT4-GFP was similarly distributed among specialized compartments and TR-containing endosomes, establishing that in C/EBPα−/− adipocytes a portion of intracellular HA-GLUT4-GFP is segregated from the TR-containing endosomes (Fig. 5B). We can express the data in Fig. 5B as a percentage of intracellular HA-GLUT4-GFP in the specialized compartment versus the TR-containing endosomes (Fig. 5C). In this analysis, we assume that IRAP-TR has access to all of the compartments containing HA-GLUT4-GFP. In C/EBPα−/− adipocytes, as is the case in 3T3-L1 adipocytes, about 40–50% of the intracellular HA-GLUT4-GFP was in the specialized compartments, and the rest was in TR-containing endosomes. Re-expression of C/EBPα in the C/EBPα−/− adipocytes did not alter the distribution of HA-GLUT4-GFP between endosomes and the specialized compartment (Fig. 5C). Thus, segregation of about half of the intracellular HA-GLUT4-GFP to compartments not accessible to the TR was independent of C/EBPα expression.

To establish that the specialized compartment is formed in the C/EBPα−/− cells upon differentiation into adipocytes, we determined the co-localization of GLUT4 and TR in C/EBPα−/− preadipocytes. In the C/EBPα−/− preadipocytes, we found complete overlap between the GLUT4 and TR compartments, confirming that, as in wild type adipocytes, the specialized GLUT4 compartment develops in C/EBPα−/− cells upon differentiation, although GLUT4 traffic through this pathway in the knockout adipocytes is not regulated (Fig. 6).

**DISCUSSION**

Our data demonstrate that GLUT4 trafficking is defective in C/EBPα−/− adipocytes, from which we conclude that C/EBPα controls the expression of proteins required for the basal, slow exocytosis of GLUT4 in adipocytes. In the absence of C/EBPα, GLUT4 accumulated on the surface of adipocytes because it was rapidly exocytosed. The high amount of GLUT4 on the plasma membrane in the basal state, in effect,
blunted the ability of insulin to cause a further increase. The basal accumulation of GLUT4 on the surface explains the previously documented high basal glucose uptake and the inability of insulin to increase glucose uptake into adipocytes lacking C/EBPα (21, 22). The insulin-regulated trafficking pathway is specifically affected by the loss of C/EBPα, since the trafficking of both IRAP and GLUT4 are affected, whereas the trafficking of the TR, a marker of general endocytic traffic, is not markedly altered.

There is a reduction in insulin receptor and insulin receptor substrate-1 amounts in adipocytes lacking C/EBPα (21). The observation that the trafficking of GLUT4 in the unstimulated state is altered in adipocytes lacking C/EBPα makes it unlikely that changes in acute response to insulin signaling are responsible for the blunted translocation of GLUT4. If the previously documented reduced amounts of insulin receptor and insulin receptor substrate-1 contribute to the altered basal trafficking of GLUT4, then they probably affect differentiation of cells in a
way that gives rise to adipocytes that are unable to retain GLUT4 in the basal state.

The molecular mechanism of GLUT4 retention in adipocytes has not been elucidated. Current models propose that GLUT4 cycles intracellularly between endosomes and a specialized compartment in basal conditions, but the rate-limiting step in exocytosis to the cell surface is not known. Considerable efforts have focused on characterizing the specialized compartment, because both the slow exocytosis of GLUT4 and its pronounced internalization by IRAP-TR or TR is plotted. The ablation was performed and indicated that expression of the latter proteins, but not the former, are

Possible. Insulin could activate a process that increases GLUT4 exocytosis by releasing the clamp on GLUT4 movement, and it is this clamp that is lacking in C/EBPα−/− adipocytes. Our observations that the GLUT4 distribution in basal C/EBPα−/− adipocytes is similar to insulin-treated adipocytes that express C/EBPα and that insulin does not increase the amount of GLUT4 on the surface of adipocytes lacking C/EBPα both support the hypothesis that insulin acts by releasing an inhibitory factor. Although our data are most consistent with insulin’s effect on GLUT4 redistribution resulting from the release of a negative regulator, they do not exclude the possibility that insulin also stimulates GLUT4 exocytosis in normal adipocytes by activating a positive regulator of GLUT4 trafficking. However, if this is the case, this activity must also be disrupted in adipocytes lacking C/EBPα. Understanding the mode of insulin action is important, because it will help frame future studies of the molecular mechanisms of insulin action on GLUT4 traffic.

Reintroduction of C/EBPα into the adipocytes lacking endogenous C/EBPα restores both basal retention of GLUT4 and an ∼3-fold insulin-stimulation of GLUT4 exocytosis. These results are in agreement with the results of a previous study that showed that reintroduction of C/EBPα reduced basal glucose uptake and restored an ∼3-fold insulin-stimulated glucose uptake (21, 30). Although there are clear, significant differences in the behavior of GLUT4 in adipocytes lacking C/EBPα and those expressing C/EBPα by retroviral infection, the magnitude of the insulin response was considerably less than that observed in 3T3-L1 adipocytes (Figs. 2 and 3). The basal exocytic rate constant we measured for GLUT4 in C/EBPα−/− adipocytes expressing C/EBPα by retroviral infection is ∼5 times faster than the exocytosis rate constant we have previously measured for HA-GLUT4-GFP in 3T3-L1 adipocytes (3). The more rapid basal exocytosis results in a greater fraction of GLUT4 on the surface of the C/EBPα−/− adipocytes expressing C/EBPα by retroviral infection. Insulin had the same quantitative effect on GLUT4 distribution in adipocytes heterozygous for C/EBPα as in C/EBPα−/− adipocytes expressing exogenous C/EBPα, confirming that exogenous expression of C/EBPα restores normal insulin responsiveness to the knockout adipocytes. Qualitative differences in insulin responsiveness of different fibroblast cell types converted to adipocytes by expression of PPARγ and/or C/EBPα have been reported, although the underlying molecular mechanism for these differences is not known (22).

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