The Adipocyte Plasma Membrane Caveolin Functional/Structural Organization Is Necessary for the Efficient Endocytosis of GLUT4*

Received for publication, August 21, 2002, and in revised form, December 19, 2002
Published, JBC Papers in Press, December 20, 2002, DOI 10.1074/jbc.M208563200

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It is well established that insulin stimulation of glucose uptake requires the translocation of intracellular localized GLUT4 protein to the cell surface membrane. This plasma membrane redistributed GLUT4 protein was partially co-localized with caveolin as determined by confocal fluorescent microscopy but was fully excluded from lipid rafts based upon Triton X-100 extractability. Cholesterol depletion with methyl-β-cyclodextrin, filipin, or cholesterol oxidase resulted in an insulin-independent increase in the amount of plasma membrane localized GLUT4 that was fully reversible by cholesterol replenishment. This basal accumulation of cell surface GLUT4 occurred due to an inhibition of GLUT4 endocytosis. However, this effect was not specific since cholesterol extraction also resulted in a dramatic inhibition of clathrin-mediated endocytosis as assessed by transferrin receptor internalization. To functionally distinguish between caveolin- and clathrin-dependent endocytic processes, we took advantage of a dominant-interfering caveolin 1 mutant (Cav1/S80E) that specifically disrupts caveola organization. Expression of Cav1/S80E, but not the wild type (Cav1/WT) or Cav1/S80A mutant, inhibited cholera toxin B internalization without any significant effect on transferrin receptor endocytosis. In parallel, Cav1/S80E expression increased the amount of plasma membrane localized GLUT4 protein in an insulin-independent manner. Although Cav1/S80E also decreased GLUT4 endocytosis, the extent of GLUT4 internalization was only partially reduced (~40%). In addition, expression of Cav1/WT and Cav1/S80A enhanced GLUT4 endocytosis by ~20%. Together, these data indicate that the endocytosis of GLUT4 requires clathrin-mediated endocytosis but that the higher order structural organization of plasma membrane caveolin has a significant influence on this process.

One of the major acute actions of insulin is enhanced glucose uptake in striated muscle and adipose tissue (1–3). This results from the rapid translocation of the intracellular-sequestered GLUT4† glucose transporter isofrom to the plasma membrane (4, 5). The increase in plasma membrane GLUT4 occurs due to a large increase in the rate of GLUT4 exocytosis coupled with a smaller decrease in the rate of GLUT4 endocytosis (6, 7). Recent data suggest that two independent signal transduction pathways are necessary for the full extent of insulin-stimulated GLUT4 translocation. In one case, the insulin receptor tyrosine phosphorylates insulin receptor substrate-family proteins, resulting in the activation of phosphatidylinositol 3-kinase and the generation of phosphatidylinositol 3,4,5-triphosphate. Although well defined, the serine/threonine kinases phosphoinositide-dependent protein kinase 1 and protein kinase B/Akt as well as protein kinase C/α have been implicated in signaling events functioning downstream of phosphatidylinositol 3-kinase (8–10). This pathway appears to be spatially segregated from a parallel insulin receptor-signaling pathway that results in the tyrosine phosphorylation of Cbl (11). In turn, Cbl is recruited to plasma membrane lipid raft microdomains through the CAP (Cbl-associated protein) adaptor protein that binds to both Cbl and the caveolar protein flotillin (12). Although substantial progress has been made in our understanding of the GLUT4 exocytotic process, the mechanisms and pathways involved in GLUT4 endocytosis and recycling are poorly understood. Several studies have indicated that GLUT4 is internalized through a clathrin-dependent endocytic pathway. For example, potassium depletion disrupts clathrin-coated pits and inhibits GLUT4 endocytosis (13). Inhibition of AP2 or dynamin function also prevents endocytosis and results in the accumulation of GLUT4 at the cell surface (14–16). Furthermore, morphological analysis demonstrates the association of GLUT4 with clathrin-coated pits, and GLUT4 appears to initially co-internalize with the transferrin receptor (17–19). Whether or not GLUT4 partitions efficiently into caveola-enriched plasma membrane fractions remains controversial. Several studies report that caveolin-enriched fractions contain the majority of GLUT4 (20–23), whereas others have not detected any association between GLUT4 and caveolin (19, 24). Nevertheless, disruption of lipid raft structure by cholesterol depletion effectively inhibits insulin-stimulated GLUT4 translocation and glucose uptake (25, 26). Furthermore, intracellular GLUT4 compartments were also observed to be devoid of caveolin, suggesting that if GLUT4 is caveolin-associated at the plasma membrane, after endocytosis GLUT4 must segregate from these caveolin-enriched domains (27). One interpretation of these data is that plasma membrane caveolin-enriched lipid raft microdomains may be involved in the insulin-stimulated GLUT4 endocytic process. Consistent with this hypothesis, cholesterol depletion has recently been reported to inhibit GLUT4 endocytosis (23). However, this interpretation is complicated because cholesterol depletion can also inhibit clathrin-mediated endocytosis through a physical restraint on membrane curvature (28, 29). Moreover, cholesterol depletion does not distinguish between cell surface caveolae versus caveolae localized to internal membrane compartments; thus it is not

* This work was supported by National Institutes of Health Research Grants DK33823, DK59291, and DK55811. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked ‘advertisement’ in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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† The abbreviations used are: GLUT4, insulin-responsive glucose transporter; Cav1, caveolin 1; MβC, methyl-β-cyclodextrin; PBS, phosphate-buffered saline; CAP, Cbl-associated protein; HA, hemagglutinin; WT, wild type; FITC, fluorescein isothiocyanate; EGFP, enhanced green fluorescent protein.

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clear if GLUT4 endocytosis has specifically been affected in these experiments.

To resolve some of these issues, we have examined the effect of cholesterol extraction and expression of caveolin mutants on the localization and rate of GLUT4 endocytosis in 3T3L1 adipocytes. Our data demonstrate that increased expression of wild type caveolin accelerates the extent of GLUT4 translocation to the plasma membrane. Insulin-stimulated expression of a dominant-interfering caveolin mutant inhibited GLUT4 endocytosis without any significant effect on clathrin-dependent internalization. However, the specific disruption of caveolin organization only partially inhibited GLUT4 endocytosis, whereas blockade of both clathrin and caveolin function resulted in a near complete block of GLUT4 internalization. These data are consistent with a model wherein caveolae and clathrin function together to mediate the efficient endocytosis of GLUT4.

EXPERIMENTAL PROCEDURES

Materials—The Myc, HA, and clathrin antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Caveolin-2 antibody was from Transduction Laboratories (Lexington, KY). Transferrin receptor antibody was from Cell Systems (Aurora, OH). The polyclonal antibody against GLUT4 (LAO) and TC10 were obtained as previously described (14, 30). Texas Red-conjugated transferrin was from Molecular Probes (Eugene, OR). Fluorescent secondary antibodies were purchased from Jackson Immunoresearch Laboratories (West Grove, PA). Cholesterol, filipin III, methyl-β-cyclodextrin, and FITC-labeled cholera toxin B were from Sigma. Cholesterol oxidase was from Calbiochem. The Myc-tagged wild type caveolin 1 (Cav1/WT) cDNA was mutated to replace serine 80 with glutamic acid (Cav1/S80E) or with alanine (Cav1/S80A) via PCR and was cloned into pcDNA3 vector (Invitrogen). GLUT4-EGFP cDNA was constructed as previously described (31), and exofacial HA-tagged GLUT4 was generated by inserting the sequence of the first exofacial loop of GLUT4 cDNA and cloning into pcDNA3 vector. Human transferrin receptor cDNA was purchased from American Type Tissue Culture (Manassas, VA) and subcloned into pcDNA3 vector.

Cell Culture and Transient Transfection by Electroporation—Murine 3T3L1 preadipocytes (American Type Tissue Culture) were grown and transfected with pcDNA3 vectors transfected into 3T3L1 preadipocytes using a low voltage electroporation protocol. The 3T3L1 adipocytes were electroporated with various cDNAs using a low voltage electroporation technique (150 V at 950 microfarads) as previously described (33). After transfection, the cells were plated on collagen-coated coverslips and incubated for 18–24 h before analysis.

Immunofluorescence Microscopy—Intact cell immunofluorescence was performed by washing the cells once with ice-cold PBS, followed by fixation with 4% paraformaldehyde (Electron Microscopy Sciences, Ft. Washington, PA) and 0.2% Triton X-100 in PBS at room temperature for 10 min. The cells were then blocked with 5% donkey serum. Plasma membrane sheets were prepared by the method of Robinson et al. (19). Briefly, the membranes were fixed in 2% paraformaldehyde at room temperature for 20 min and blocked with 5% donkey serum. The cells or plasma membrane sheets were then incubated with primary antibodies for 90 min at 37 °C and Texas Red- or FITC-conjugated donkey secondary antibody for 2 h at room temperature. The coverslips were mounted in Vectashield (Vector Laboratories, Inc. Burlington, CA) and examined with 40× or 63× oil immersion objectives using a Zeiss 510 confocal laser-scanning microscope.

Drug Treatment—Fully differentiated 3T3L1 adipocytes were pretreated with 10 mM methyl-β-cyclodextrin, 5 μM filipin III, or 2 units/ml cholesterol oxidase in serum starvation medium for the indicated period and then stimulated by 100 nM insulin for 30 min. To examine the effect of cholesterol recovery, cholesterol-methyl-β-cyclodextrin complexes were synthesized as described previously (34). Briefly, cholesterol methyl carbonate was dissolved in 12.6 μl of dichloromethane (CHCl3, 2:1) solution. Methyl-β-cyclodextrin (31.5 mg) was dissolved in 345.6 μl of double-distilled H2O and heated to 80 °C with stirring. The cholesterol was added to methyl-β-cyclodextrin, and the solution was stirred until clear. This solution contained 6.8 mM cholesterol. For use, complexes were diluted into serum starvation medium to a final concentration of 0.2% (w/v). After methyl-β-cyclodextrin treatment, the cells were incubated with 0.2 μM cholesterol-methyl-β-cyclodextrin complexes for the indicated period.

GLUT4 Endocytosis Assay—3T3L1 adipocytes co-transfected with pcDNA3 vector, Myc-Cav1/WT, Myc-Cav1/S80E, or Myc-Cav1/S80A and exofacial HA-tagged GLUT4 were insulin-stimulated for 30 min at 37 °C. Then the cells were chilled and incubated with HA monoclonal antibody for 1 h at 4 °C to label the GLUT4 at the plasma membrane, and cells were washed to remove insulin and excess HA antibody as described previously (14). The cells were returned to 37 °C and incubated for various times to allow HA antibody-bound GLUT4 to internalize. The reactions were stopped by washing once with ice-cold PBS and fixing in 4% paraformaldehyde and 0.2% Triton X-100 in PBS at room temperature for 10 min. The cells were incubated with Myc polyclonal antibody followed by Texas Red-anti-mouse and FITC-anti-rabbit secondary antibody. Translocation of HA antibody-bound GLUT4 from plasma membrane to the intracellular pool was examined by immunofluorescence microscopy.

Transferrin Receptor Endocytosis—3T3L1 adipocytes were overexpressing human transferrin receptor were pretreated with or without 10 μM methyl-β-cyclodextrin for 30 min. Then the cells were incubated with 5 μg/ml Texas Red-conjugated transferrin for 4 °C for 1 h to label the surface transferrin receptor followed by washing the cells 4 times with ice-cold PBS. The cells were returned to 37 °C and incubated for the indicated period to allow the endocytosis of labeled transferrin receptor. Then transferrin receptor endocytosis was examined by a confocal laser-scanning microscope.

Cholera Toxin B Uptake—Cholera toxin B uptake was examined as described previously (35). Briefly, differentiated 3T3L1 adipocytes were rinsed twice with Hanks’ balanced salt solution and serum-starved for 2 h. Then the cells were chilled and incubated with 4 μg/ml FITC-labeled cholera toxin B for 30 min at 4 °C. The cells were washed 4 times, returned to 37 °C, and incubated for 2.5 h. Uptake of FITC-labeled cholera toxin B was examined by a confocal laser-scanning microscope.

Triton X-100 Extraction and Immunoblot—Plasma membrane sheets of 3T3L1 adipocytes were extracted with a lysis buffer containing 25 mM Hepes, pH 7.4, 150 mM NaCl, 1% Triton X-100, 50 mM sodium fluoride, 10 mM sodium pyrophosphate, 1 mM sodium vanadate, 10 μg/ml aprotinin, 1 μg/ml pepstatin, 5 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride for 15 min at 0 °C. The samples were then centrifuged at 16,000 × g for 10 min, the supernatants were collected, and the pellets were washed once with ice-cold PBS, re-centrifuged, and re-suspended in the lysis buffer. The supernatants and re-suspended pellets were placed in Laemmli sample buffer, and the proteins were separated in a 7.5–20% gradient SDS-polyacrylamide gel, transferred to a polyvinylidene difluoride-blotting membrane (Millipore Corp., Bedford, MA), and analyzed for transferrin receptor, GLUT4, and caveolin by immunoblotting.

RESULTS

Plasma Membrane-localized GLUT4 Partially Associates with Large Caveolin Clusters—Previously, we and others have observed that differentiated 3T3L1 adipocytes assemble large clusters of individual caveolae (caveolae rosettes) that are discernible by confocal fluorescent microscopy (25, 30, 36). Consistent with our previous findings (30), the lipid raft-targeted small GTP-binding protein TC10 and caveolin co-localize with these plasma membrane structures (Fig. 1, A, panels a–c). However, the organization of these proteins is distinct from marker proteins that do not associate in lipid raft microdomains such as the transferrin receptor, which recycles from the plasma membrane through clathrin-coated pits, and the clathrin coat protein itself (Fig. 1A, panels d–i). In the basal state, caveolin displays its characteristic cluster organization that is apparent at both low and high magnifications (Fig. 1B, panels e and f). However, in the absence of insulin the amount of GLUT4 protein present in the plasma membrane is very low, and thus, there is essentially no colocalization with caveolin (Fig. 1B, panels a, b, e, f, i, and j). Insulin stimulation had no significant effect on the organization of caveolin, but there was a marked increase in the plasma membrane association of GLUT4 (Fig. 1B, panels c, d, g, h, k, and l). Comparison of these images indicated that GLUT4 was partially dispersed throughout the plasma membrane. However, GLUT4 did display some co-localization with the caveolin-positive ring structures, although not nearly as pronounced as the TC10 marker.

Because the apparent association of GLUT4 with the large
clusters of organized caveolin was indeterminate, we assessed the association of GLUT4 with lipid raft microdomains by cold Triton X-100 extraction (Fig. 2). Total Triton X-100 extracts of isolated plasma membrane sheets (lysates) demonstrated the presence of the transferrin receptor, GLUT4, and caveolin (Fig. 2, lane 1). After centrifugation, the resulting soluble fraction contained all three proteins, whereas the insoluble pellet fraction was completely devoid of the transferrin receptor and GLUT4 (Fig. 2, lanes 2 and 3). Although a majority of the caveolin 1 protein was found in the soluble fraction, there was a substantial amount resistant to cold Triton X-100 extraction. As expected, insulin stimulation demonstrated a large increase in the amount of plasma membrane sheet-localized GLUT4 along with a smaller increase in the translocation of the transferrin receptor (Fig. 2, lanes 4 and 5). Nevertheless, both the transferrin receptor and GLUT4 remained completely cold Triton X-100-soluble, whereas caveolin 1 was only partially extracted (Fig. 2, lanes 5 and 6). Together, these data demonstrate that GLUT4 is not an integral component of caveolin-containing lipid raft microdomains in 3T3L1 adipocytes.

Cholesterol Depletion Inhibits GLUT4 Endocytosis—Recently several studies have reported that cholesterol extraction can inhibit insulin-stimulated glucose uptake (23, 25, 26). To determine whether this resulted from an increase in plasma membrane-localized GLUT4, we next treated adipocytes with several agents known to alter lipid raft microdomains through modification of cell surface cholesterol (Fig. 3). As expected, there was essentially no detectable plasma membrane-associated GLUT4 protein in the basal state, but after insulin stimulation GLUT4 was readily apparent (Fig. 3A, panels a and e). Treatment with methyl-β-cyclohextrin (MβCD) and cholesterol (Chol) oxidase resulted in an increase in the cell surface GLUT4 protein levels and did not display any further statistically significant increase by insulin (Fig. 3A, panels b, d, f, and h). Although filipin also increased the basal level of plasma membrane-associated GLUT4, insulin was still capable of inducing a further stimulation (Fig. 3A, panels c and g). These results were quantitated by determining the relative fluorescent intensity of plasma membrane sheets from three independent experiments (Fig. 3B).

To ensure that this was a specific effect of cholesterol depletion, adipocytes were first treated with MβCD and subsequently repleted with cholesterol (Fig. 4). As is readily apparent, MβCD treatment resulted in the cell surface appearance of GLUT4 that was fully reversed by re-introduction of cholesterol in a time-dependent manner (Fig. 4, panels a–f). Together, these data demonstrate that cholesterol depletion results in the accumulation of GLUT4 protein at the plasma membrane. The increase in plasma membrane GLUT4 could result from...
HA-GLUT4 was strongly labeled at the cell surface with essentially no interior labeling at 4 °C (Fig. 5A, panels a and e). In untreated cells, there was a rapid time-dependent internalization of GLUT4 that concentrated in small compartments beneath the plasma membrane and in the perinuclear region (Fig. 5A, panels b–f). Pretreatment of the cells with MβCD resulted in a marked reduction in the time-dependent appearance of intracellular-localized GLUT4 (Fig. 5A, panels f–h).

It should be noted that although MβCD treatment decreased the rate of GLUT4 internalization, it was not completely inhibited. This is better exemplified by plotting the number of cells displaying internalized GLUT4 after insulin removal (Fig. 5B).

Several studies report that cholesterol depletion can impair both lipid raft- and non-lipid raft-dependent internalization (28, 29). To address this issue in our experimental system, we also determined the effect of MβCD treatment on the internalization of the transferrin receptor (Fig. 5C). After cell surface labeling at 4 °C and warming to 37 °C, the exofacial labeled transferrin receptor internalized with a similar rate and extent as GLUT4 in untreated cells. Similarly, the rate and extent of internalized transferrin receptor was substantially reduced in cells pretreated with MβCD. Thus, the impairment of GLUT4 endocytosis by cholesterol depletion accounts for its accumulation at the plasma membrane. However, these data do not distinguish whether GLUT4 endocytosis occurs through either a clathrin-dependent and/or lipid raft-dependent mechanism.

Expression of Caveolin 1 Mutants Specifically Inhibits Lipid Raft-dependent Endocytosis—Caveolin 1 can undergo phosphorylation at Ser-80 that results in the intracellular retention of caveolin 1 and co-sequestration of caveolin 2 (37, 38). In turn, the loss of cell surface caveolin 1 and two proteins prevents the formation of caveolae structures and impairs caveolin-dependent functions. Thus, to distinguish between caveolin-dependent and non-caveolin-mediated endocytosis, we tested the ability of caveolin 1 mutants to affect caveolin organization and function in adipocytes (Fig. 6). The expressed wild type caveolin 1 (Cav1/WT) protein was distributed in ring-like clustered patterns on the plasma membrane that co-localized with endogenous caveolin 2 (Fig. 6, panels a and d). In contrast, expression of the caveolin 1 mutant (Cav1/S80E) that mimics phosphorylation disrupted the organized plasma membrane caveolin clusters, whereas the Cav1/S80A mutant was without any significant effect (Fig. 6, panels b, c, e, and f).

To determine whether Cav1/S80E impaired caveolin-dependent endocytosis, we assessed the internalization of the cholera toxin B subunit (Fig. 7). It has been well established that the endocytosis of cholera toxin B occurs through binding to the lipid raft/caveolae-associated glycolipid GM1 (39, 40, 53). Adipocytes transfected with the empty vector were first incubated at 4 °C with cholera toxin B (CT-B) and then warmed to 37 °C for 2.5 h. Under these conditions, cholera toxin B was effectively internalized in the empty vector control-transfected cells (Fig. 7A, panels a and e). Similarly, expression of Cav1/S80A had no significant effect on the intracellular accumulation of cholera toxin B (Fig. 7A, panels d and h). In contrast, cells expressing Cav1/WT displayed a small increase in cholera toxin B endocytosis, whereas Cav1/S80E had a reduction in the amount of internalized cholera toxin B (Fig. 7A, panels b, c, f, and g).

Quantitation of the number of cells displaying internalized cholera toxin B are presented in Fig. 7B. In contrast to cholera toxin B, transferrin receptor endocytosis was essentially identical in cells co-expressing the empty vector, Cav1/WT, Cav1/S80E, and Cav1/S80A (Fig. 7C). These data demonstrate that unlike MβCD, Cav1/S80E specifically inhibits caveolin-dependent endocytosis without affecting clathrin-dependent endocytosis.

### Figure 3.

**Cholesterol depletion results in an insulin-independent accumulation of cell surface GLUT4 protein.** A, 3T3L1 adipocytes were incubated in the absence (panels a and e) or presence of 10 mM MβCD (panels b and f), 5 μg/ml filipin (panels c and g), or 2 μg/ml cholesterol oxidase (Chol Oxidase, d and h) for 30 min. The cells were then treated without (panels a–d) or with (panels e–h) 100 nM insulin for 30 min. Plasma membrane sheets were prepared and examined for the distribution of GLUT4 by confocal fluorescent microscopy. These are representative images from three independent determinations. B, the relative extent of GLUT4 translocation was quantified by determining the relative fluorescent intensity of the plasma membrane sheets.

### Figure 4.

**Cholesterol repletion reverses the insulin-independent plasma membrane accumulation of GLUT4 after cholesterol depletion.** 3T3L1 adipocytes were incubated in the absence (panel a) or presence of 10 mM MβCD (panel b) for 30 min. The cells that were MβCD-treated were then incubated with a cholesterol-MβCD complex for 10 (panel c), 30 (panel d), 60 (panel e), and 120 (panel f) min before the preparation of plasma membrane sheets and examined for the distribution of GLUT4 by confocal fluorescent microscopy. These are representative images from three independent determinations. SFM, serum-free medium.

Either an increase in exocytosis and/or decrease in endocytosis. Because previous studies report that cholesterol extraction can inhibit endocytosis (28, 29), we determined the effect of MβCD on GLUT4 and transferrin receptor internalization (Fig. 5). Adipocytes were transfected with an exofacial HA epitope-tagged GLUT4 and stimulated with insulin to induce GLUT4 translocation and accumulation of the HA epitope on the plasma membrane. After HA antibody labeling at 4 °C, the insulin was removed by extensive washing, and the cells were then warmed to 37 °C for various times. As is apparent, the
Having established a method to resolve clathrin- and caveolin-dependent internalization, we next determined the effect of caveolin expression on insulin-stimulated GLUT4 translocation (Fig. 8). In adipocytes co-transfected with an empty vector and GLUT4-EGFP, insulin stimulated the translocation of the GLUT4-EGFP reporter from intracellular storage sites to the plasma membrane. Expression of Cav1/WT and Cav1/S80A had no significant effect on either the basal state distribution or insulin-stimulated GLUT4-EGFP translocation. In contrast, expression of Cav1/S80E resulted in a marked increase in the basal accumulation of GLUT4-EGFP at the plasma membrane that was not further stimulated by insulin.

Analysis of GLUT4 endocytosis was then performed using the exofacial HA epitope-tagged GLUT4 as previously described in Fig. 5. Compared with vector-transfected cells, expression of Cav1/S80E significantly reduced the extent of GLUT4 endocytosis (Fig. 9). Importantly, the Cav1/S80E inhibition was only partial, and the initial phase of GLUT4 endocytosis appeared to be unaffected. In addition, expression of Cav1/WT and Cav1/S80A appeared to enhance the extent of GLUT4 endocytosis compared with the empty vector-transfected control cells. Together these data demonstrate that the initial rate of GLUT4 endocytosis occurs through a clathrin-mediated process but that caveolin function is necessary for the full extent of GLUT4 internalization.

**DISCUSSION**

Lipid rafts are membrane microdomains that are enriched in cholesterol and glycosphingolipids that can form a relatively ordered and stable liquid phase within the more fluid, disordered structure of phospholipid bilayers (41, 42). Adipocytes are one of the most abundant sources of a particular subtype of lipid raft called caveolae that accounts for a large amount of the plasma membrane surface (20, 24, 43). Caveolae are characterized by the presence of the caveolin coat protein that forms fl- or flask-shaped invaginations (44). In adipocytes, many of these individual caveolae are organized into large ring-like clusters (caveolae-rosettes) that are visible by light microscopy (25, 30, 36). Numerous studies demonstrate that lipid raft domains play an important role in the spatial compartmentalization of various signaling proteins, effector functions, and for...
specific membrane-trafficking events (45, 46). For example, caveolae are involved in the specific assembly of receptor and non-receptor tyrosine kinase-signaling complexes as well as trimeric and small GTP-binding proteins. In addition, these domains are involved in potocytosis, transcytosis, clathrin-independent endocytosis, and bacterial entry (42, 45).

Recently, several studies have also implicated caveolin-enriched lipid raft microdomains in the insulin regulation of GLUT4 translocation (11, 12, 23, 25, 26, 30). The insulin receptor has been reported to associate with caveolin and to generate a novel signaling cascade involving the lipid raft recruitment and tyrosine phosphorylation of the APS/Cbl/CAP complex (11, 47–50). This pathway appears to function in concert with the insulin receptor tyrosine phosphorylation and activation of the insulin receptor substrate/phosphatidylinositol 3-kinase pathway in mediating the full extent of GLUT4 translocation (12). Although controversial, after insulin stimulation GLUT4 has been observed to co-localize with caveolin by FIG. 7. Expression of Cav1/S80E inhibits cholera toxin B but not transferrin receptor internalization. A, 3T3L1 adipocytes were transfected with the empty vector (pcDNA3) or vector encoding Myc epitope-tagged Cav1/WT, Cav1/S80E, and Cav1/S80A mutants. Twenty-four h later, the cells were cooled to 4 °C, incubated for 30 min with 4 μg/ml FITC-labeled cholera toxin B (CT-B), and warmed to 37 °C for 2.5 h. The cells were then fixed and subjected to confocal fluorescent microscopy for the presence of the Myc epitope and FITC-cholera toxin B internalization. These are representative images obtained from two independent determinations. B, the amount of internalized cholera toxin B was quantified by counting the number of cells displaying intracellular labeling relative to control vector-transfected cells. These data were obtained from the counting of a total of 80 cells from 2 independent experiments. C, in parallel, the transfected cells were cooled to 4 °C and labeled with Texas Red-labeled transferrin and warmed to 37 °C for the times indicated. TfR, transferrin receptor. These data were obtained from the counting of 40 cells per experiment from 3 independent determinations.

FIG. 8. Expression of Cav1/S80E results in the insulin-independent accumulation of GLUT4 at the plasma membrane. 3T3L1 adipocytes were co-transfected with the GLUT4-EGFP cDNA and either the empty vector (pcDNA3) or vectors encoding Myc epitope-tagged Cav1/WT, Cav1/S80E, and Cav1/S80A mutants. Twenty-four h later, the cells were incubated in the absence (open bars) or presence (closed bars) of 100 nM insulin for 30 min at 37 °C. The cells were then fixed and subjected to confocal fluorescent microscopy for the presence of the Myc epitope and the localization of GLUT4-EGFP. These data were obtained from the counting of 40 cells per experiment from 4 independent determinations.

FIG. 9. Expression of Cav1/S80E inhibits, whereas expression of Cav1/WT and Cav1/S80A stimulates, GLUT4 endocytosis. 3T3L1 adipocytes were co-transfected with the HA-GLUT4 cDNA and either the empty vector (pcDNA3) or vectors encoding Myc epitope-tagged Cav1/WT, Cav1/S80E, and Cav1/S80A mutants. Twenty-four h later, the cells were incubated with 100 nM insulin for 30 min at 37 °C and then cooled to 4 °C. The cells were then incubated with the HA antibody for 60 min and extensively washed to remove the insulin and unbound antibody. The cells were warmed to 37 °C for the times indicated, and confocal fluorescent microscopy was performed for the presence intracellular localization of the HA and Myc epitopes. These data were obtained from the counting of 40 cells per experiment from 3 independent determinations.
immuno-electron microscopy, fluorescent microscopy, sucrose gradient flotation, and Triton X-100 extractability (20–23). Furthermore, cholesterol depletion and disruption of caveolae by MβCD treatment has been found to block insulin-stimulated GLUT4 translocation and glucose uptake (23, 25, 26). However, the interpretation of these latter findings is difficult because MβCD can also prevent clathrin-mediated endocytosis as well as the insulin receptor activation and recruitment of APS/Chi/CAP-signaling complex (23, 25, 26, 28, 29). Indeed, our data also demonstrated that cholesterol depletion with MβCD, filipin, and cholesterol oxidase all increased the plasma membrane content of GLUT4 in an insulin-independent manner. These data are consistent with other studies demonstrating that inhibition of endocytosis results in an accumulation of GLUT4 at the plasma membrane. However, although MβCD markedly inhibited GLUT4 endocytosis, it was also a potent inhibitor of transferrin receptor internalization, an established marker for clathrin-mediated endocytosis. Thus, this type of pharmacological approach does not necessarily distinguish between caveolin- and non-caveolin-dependent events.

To more directly assess the specific role that caveolin/lipid rafts may play in GLUT4 recycling, we took advantage of a caveolin 1 mutation that mimics phosphorylation on serine 80 (Cav1/S80E). Previous studies report that in the unphosphorylated state caveolin 1 assembles into plasma membrane caveolae along with caveolin 2 (37, 38). However, when phosphorylated on Ser-80, caveolin 1 is retained within the endoplasmic reticulum in a complex with caveolin 2, and this phenotype is recapitulated with the Cav1/S80E mutant. As expected, expression of Cav/S80E in adipocytes, but not Cav1/WT or Cav1/S80A, disrupted the caveolae rosette organization and inhibited the internalization of cholela toxin B, an established marker for lipid raft-localized GM1-dependent endocytosis (40). More importantly, Cav1/S80E had no significant effect on transferrin receptor internalization, demonstrating that clathrin-mediated endocytosis was not perturbed under these conditions. Thus, the expression of Cav1/S80E provided an experimental tool to distinguish between caveolin- and clathrin-dependent endocytic mechanisms.

Using this approach, an examination of GLUT4 distribution revealed an insulin-independent plasma membrane accumulation of GLUT4 in cells expressing Cav1/S80E but not Cav1/WT or Cav1/S80A. Furthermore, Cav1/S80E reduced the extent of GLUT4 endocytosis, whereas Cav1/WT and Cav1/S80A enhanced GLUT4 endocytosis. These data are consistent with caveolin functioning in the GLUT4 endocytosis process. Importantly, the effect of Cav1/S80E was only partial and primarily affected the extent but not the initial rate of GLUT4 endocytosis. Similarly, Cav1/WT and Cav1/S80A increased the extent but not the initial rate of GLUT4 endocytosis. These findings suggest that the initial rate of GLUT4 endocytosis occurs through a clathrin-dependent pathway and accounts for greater than 50% of the cell surface GLUT4 protein. The remaining 30–40% of the plasma membrane GLUT4 protein is either slowly internalized through a caveolin-dependent vesicle endocytosis or alternatively passes through a caveolin-organized structural domain before entry into a clathrin-coated pit.

The hypothesis that GLUT4 internalization occurs via two distinct and/or sequential pathways can reconcile several of the discrepancies in the literature. Multiple studies utilize immunoelectron microscopy, Triton X-100 extractability, and sucrose gradient flotation to assess the co-localization of GLUT4 with caveolin and provide both evidence for and against caveolin association (19–24, 51). These apparent contradictory findings could result if different experimental manipulations favored one GLUT4 population over the other. For example, GLUT4 could exist in an equilibrium between caveolin and non-caveolin regions of the plasma membrane and, therefore, may be concentrated in one compartment depending upon the specific conditions used for detergent extractions and/or fixation. Alternatively, the fraction of GLUT4 that appears to undergo caveolin-dependent endocytosis may not actually be within individual caveolae structures but might be loosely associated around the periphery of the large plasma membrane caveolin domains. Although we have no direct evidence for this, we favor this latter possibility based upon several observations. For example, proteins that are clearly caveolin-associated (e.g. TC10) display a very strong and distinct co-localization with the caveolin ring structures. In contrast, GLUT4 is weakly co-localized near these domains and only partially overlaps with caveolin. Furthermore, isolation of highly purified adipocyte caveolae did not reveal the presence of GLUT4, demonstrating that GLUT4 is not embedded within this type of lipid raft structure. In addition, recent electron microscopic analysis also suggests that insulin recruits GLUT4 to large cave-like structures that contain caveolin in addition to clathrin-coated pits, lipid raft, and non-lipid raft markers (52). However, although GLUT4 appeared to be peripherally associated with these large caveolin structures there was no specific co-localization of GLUT4 within individual caveolae. This is consistent with our cold Triton X-100 extraction results indicating that GLUT4 is not directly embedded in lipid raft microdomains.

Thus, we propose a model in which GLUT4 is distributed between non-caveolin regions of the plasma membrane and in peripheral association with large caveolin-containing domains. The non-caveolin regions recycle more rapidly and internalize through a clathrin-dependent pathway, whereas the peripheral caveolin-associated GLUT4 undergoes a slower redistribution into clathrin-coated pit domains. Future studies are now necessary to determine whether these two compartments are in equilibrium with each other, to determine the pathways that account for the trafficking and segregation of these two distinct GLUT4 populations, and to determine how the higher order caveolin domains can modulate the functions of the non-lipid raft regions of the plasma membrane.

Acknowledgment—We thank Amanda Kalen for care and maintenance of 3T3L1 adipocytes.

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