Induction of Caveolin during Adipogenesis and Association of GLUT4 with Caveolin-rich Vesicles

Philipp E. Scherer,* Michael P. Lisanti,* Giulia Baldini,* Massimo Sargiacomo,* Cynthia Corley Mastick,‡ and Harvey F. Lodish*§

*Whitehead Institute for Biomedical Research, Cambridge, Massachusetts 02142-1479; ‡Department of Signal Transduction, Parke-Davis Pharmaceutical Research Division, Warner-Lambert Co., Ann Arbor, Michigan 48105; §Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139

Abstract. Caveolae, also termed plasmalemmal vesicles, are small, flask-shaped, non-clathrin–coated invaginations of the plasma membrane. Caveolin is a principal component of the filaments that make up the striated coat of caveolae. Using caveolin as a marker protein for the organelle, we found that adipose tissue is the single most abundant source of caveolae identified thus far. Caveolin mRNA and protein are strongly induced during differentiation of 3T3-L1 fibroblasts to adipocytes; during adipogenesis there is also a dramatic increase in the complexity of the protein composition of caveolin-rich membrane domains. About 10–15% of the insulin-responsive glucose transporter GLUT4 is found in this caveolin-rich fraction, and immuno-isolated vesicles containing GLUT4 also contain caveolin. However, in non-stimulated adipocytes the majority of caveolin fractionates with the plasma membrane, while most GLUT4 associates with low-density microsomes.

Upon addition of insulin to 3T3-L1 adipocytes, there is a significant increase in the amount of GLUT4 associated with caveolin-rich membrane domains, an increase in the amount of caveolin associated with the plasma membrane, and a decrease in the amount of caveolin associated with low-density microsomes. Caveolin does not undergo a change in phosphorylation upon stimulation of 3T3-L1 adipocytes with insulin. However, after treatment with insulin it is associated with a 32-kD phosphorylated protein. Caveolae thus may play an important role in the vesicular transport of GLUT4 to or from the plasma membrane. 3T3-L1 adipocytes offer an attractive system to study the function of caveolae in several cellular trafficking and signaling events.

C AVEOLAE, also termed plasmalemmal vesicles, are small, flask-shaped, non-clathrin–coated invaginations of the plasma membrane. Their discovery dates back to the early 1950's, when both Yamada and Palade began to study the ultrastructure of the plasma membrane in detail (6a, 55). Despite their characteristic appearance on the surface of a wide variety of cells, their precise function(s) are unknown. Recently, however, this organelle has been implicated in a variety of processes: caveolae may participate in transcytosis of both large and small molecules across capillary endothelial cells (44). Glycosylphosphatidylinositol (GPI)[1]-linked proteins on the cell surface are localized in caveolae (41, 57) though antibodies used to immunolocalize these proteins may induce clustering (34). Some of these proteins are receptors for ligands (such as folate) that seem to be internalized through caveolae, the process termed potocytosis (1). Other roles of caveolae may include transmembrane signaling, since they contain a number of cell-surface receptor and signal transducing proteins, as judged both by morphological (9, 22, 36, 38, 49) and biochemical criteria (9, 29, 30, 42).

Caveolin (also termed VIP-21), a 22-kD protein, was initially identified as a major phospho-protein in v-Src transformed cells (20). Subsequently, caveolin was shown to be an integral part of the striated coat of caveolar structures and can therefore serve as a marker for the organelle (40).

Our major interest is to understand how insulin stimulates glucose transport in fat and muscle cells. In rat adipocytes, glucose uptake increases 20–30-fold in the presence of insulin. In 3T3-L1 adipocytes, this stimulation is somewhat less, up to about 10-fold. Glucose transport is mediated by the sodium-independent glucose transporters GLUT1 and GLUT4, which, in response to insulin, translocate from an intracellular compartment to the plasma membrane (26, 45). GLUT4, which is expressed only in fat and skeletal and

1. *Abbreviations used in this paper:* GPI, glycosylphosphatidylinositol; LDM, low-density microsomal compartment.

cardiac muscle, is the primary transporter involved in this process, and is the predominant transporter expressed in these tissues (15, 53). The components mediating this translocation as well as the subsequent internalization of the transporter after removal of the insulin stimulus have remained elusive. 3T3-L1 adipocytes (4, 32, 45) are an excellent model system to study these processes. These cells are propagated in cell culture as fibroblasts. Upon addition of insulin, dexamethasone, and other factors, they differentiate into mature adipocytes over a period of eight days. Thus, the expression of proteins of interest can be monitored during the course of differentiation.

Caveolae have been observed at the morphological level in adipocyte plasma membranes. Orci and colleagues reported that during adipocyte differentiation of 3T3-L1 cells there is a nine-fold increase of structures resembling caveolae in the plasma membrane with no increase in the number of larger, clathrin-coated invaginations (16). As there has been no biochemical characterization of these organelles in these cells, we were prompted to evaluate the expression of caveolin in 3T3-L1 adipocytes. In accordance with these morphological observations, we found that both 3T3-L1 adipocytes as well as fat tissue in situ express an extremely high level of caveolin—higher than any other tissue. Caveolin is also abundant in other insulin-sensitive tissues (muscle and diaphragm) as well as in the lung, a tissue rich in endothelial cells. Our experiments suggest that in the adipocyte caveolae may be involved in the insulin-stimulated exocytosis of GLUT4 or other intracellular proteins to the plasma membrane, or in their subsequent endocytosis.

Materials and Methods

Materials

DME tissue culture medium lacking methionine, cysteine, and glutamate was purchased from ICN Biomedicals (Irvine, CA), and DME lacking phosphate and pyruvate was purchased from Specialty Media Inc. (Lavallette, NJ). Ortho-phosphate [^{32}P] was purchased from ICN Biomedicals. The express protein labeling reagent, a mixture of [^{35}S] labeled methionine and cysteine, was purchased from New England Nuclear (Boston, MA). We generated the cDNA for canine caveolin by PCR, and confirmed its identity by sequencing (42). Anti-caveolin antibodies were purchased from Transduction Laboratories (Lexington, KY). Antibodies against the α_2-subunit of Na$^+$/K$^+$-ATPase (rabbit polyclonal antiserum) were purchased from Upstate Biotechnology Incorporated (Lake Placid, NY). Identical results were obtained with antiserum McB2 which is a monoclonal mouse antibody specific for the α_2-subunit of Na$^+$/K$^+$-ATPase (52). This antiserum was a gift from K. J. Sweadner (Massachusetts General Hospital, Boston, MA). The GLUT4-specific antibody was raised against the COOH-terminal 16 amino acids of GLUT4 (3). The antiserum raised against highly purified rat outer-mitochondrial proteins was a gift of Dr. G. Schatz (University of Basel, Basel, Switzerland). The LAMP1 antibodies were a gift of Dr. H. Rosen (Merck Research Laboratories, Rahway, NJ).

Cell Culture

3T3-L1 mouse fibroblasts (American Type Culture Collection, Rockville, MD) were propagated and differentiated according to the protocol described in reference 17.

Northern Blot Analysis

Isolation of mRNA from tissues and from 3T3-L1 cells at various stages of differentiation was as described by (2), as was agarose gel electrophoresis of mRNA and its transfer to nylon membranes. Hybridizations were performed overnight at 42°C in 50% formamide, 5× SSC, 25 mM Na-phosphate, pH 7.0, 10× Denhardt's solution, 5 mM EDTA, 1% SDS, and 0.1

mg/ml Poly A; the DNA probes were used at concentrations of 2 × 10^6 cpm/ml. The filters were subsequently washed in 2× SSC/0.1% SDS and 0.1× SSC/0.1% SDS at 50°C.

Metabolic Labeling of Adipocytes

Steady state labeling of cells was performed for 12 h in a medium containing 10% normal DME and 90% DME lacking methionine and cysteine and supplemented with 0.5 mCi (1,000 Ci/mmol) of express protein labeling reagent per 10 cm dish. For in vivo phosphorylation experiments, cells were washed twice in DME lacking phosphate, incubated for 2 h at 37°C, supplemented with 1 mCi [^{32}P]orthophosphate, and then incubated an additional 3 h at 37°C. To study the effects of insulin, 100 nM insulin was added for 6 min after the labeling period.

Isolation of Caveolin-enriched Membrane Domains

Isolation of caveolin-rich membrane domains was performed according to an established method (5, 30, 42) with minor modifications. Briefly, 3T3-L1 cells (before or after differentiation) were washed three times with ice-cold PBS, scraped off the plate in 2 ml MES buffer (25 mM Mes pH 6.5, 150 mM NaCl) containing 1% Triton X-100, 1 mM PMSF, 1 μg/ml leupeptin, and homogenized 10 times in a glass Dounce homogenizer. The extract was adjusted to 40% sucrose by addition of 2 ml of an 80% sucrose solution in MES buffer, transferred to an ultracentrifuge tube, and overlaid with 8 ml of a linear 5–30% sucrose gradient in MES buffer containing PMSF and leupeptin at the above concentrations, but no Triton X-100. The gradients were centrifuged for 16 h at 4°C in a Beckman SW41 rotor at 39,000 rpm. Caveolin-enriched membranes fractionate as a sharp band at 15–20% sucrose. Equal volumes of each fraction were analyzed for SDS-PAGE and Western blotting. Alternatively, an equal volume of MES-buffer was added to caveolar fractions; the caveolin-rich domains were pelleted in a microcentrifuge and analyzed by SDS-PAGE.

Gel Electrophoresis

SDS-PAGE (28), fluography (8), densitometric scanning of gels (31), silver staining of protein gels (35), and protein determinations (BCA assay; Pierce Chemical Co., Arlington Heights, IL) were performed according to standard protocols.

Immunoprecipitations

Cells or isolated caveolae were lysed in IP buffer (1% Triton X-100, 60 mM octyl-glucoside, 150 mM NaCl, 20 mM Tris pH 8.0, 2 mM EDTA, 50 mM NaF, 30 mM Na-pyrophosphate, 100 μM Na-ortho-vanadate, 1 mM PMSF, and 2 μg/ml leupeptin). Lysates were pre-cleared by addition of 50 μl of a 1:1 slurry of protein A-Sepharose (Pharmacia Inc., Uppsala, Sweden) in TNET buffer (1% Triton X-100, 150 mM NaCl, 2 mM EDTA, 20 mM Tris pH 8.0) containing 1 mg/ml bovine serum albumin. After 30 min at 4°C, samples were centrifuged for 5 min at 15,000 g, the supernatant was transferred to a fresh tube and 50 μl protein A-Sepharose was added together with the corresponding antiserum. Samples were then incubated for 3 h at 4°C. Immunoprecipitates were washed five times in TNET and analyzed by SDS-PAGE.

Preparation of Tissue Extracts

Approximately 200 mg of various mouse tissues was lysed in 2 ml of IP buffer and homogenized on ice with a Polytron homogenizer for 30 s. Insoluble material was removed by centrifugation and the supernatant was mixed with an equal volume of 3× SDS-PAGE sample buffer. Protein was determined by the BCA method before the addition of sample buffer. Equal amounts (100 μg) were loaded for SDS-PAGE. The gel was processed for immunoblotting as described below.

Electron Microscopy

For transmission-EM, samples were fixed with glutaraldehyde, post-fixed with OsO$_4$, and stained with uranyl acetate and lead citrate, as described in reference 42.

Subcellular Fractionation and Immunoadsorption of GLUT4 Vesicles

Cell fractionation and isolation of GLUT4 vesicles of adipocytes from

epididymal fat pads of Sprague-Dawley rats was performed as described (7). Subcellular fractionation of 3T3-L1 adipocytes was as detailed in reference 3.

Immunoblotting

After SDS-PAGE, samples were transferred to a BA83 Nitrocellulose membrane (Schleicher & Schuell, Keene, NH) according to the procedure of reference 51. It is crucial to use nitrocellulose with 0.22 μm pores, since caveolin is retained inefficiently on 0.45 μm pore BA85 membranes. Primary antibodies were used in PBS containing 3% nonfat dry milk, 0.1% Tween-20. Bound antibody was visualized with the ECL system as recommended by the manufacturer (Renaissance Kit, New England Nuclear).

Results

Adipose Tissue Is a Very Rich Source of Caveolin, a Marker Protein for Caveolae

Caveolin is a principal component of the filaments that comprise the striated coat of caveolae (40), and thus the tissue distribution of caveolin should reflect the relative abundance of this organelle. In line with previous findings (21), lung and muscle are tissues rich in caveolin messenger RNA. However, the most abundant source of caveolin mRNA is white adipose tissue (Fig. 1 A, top), consistent with morphological evidence suggesting that differentiated 3T3-L1 adipocytes are a rich source of caveolae (16). Normalized to the constitutively expressed mRNA encoding cytosolic hsp70 (Fig. 1 A, bottom) (25), adipose tissue expresses four times more caveolin mRNA than lung. A longer exposure of the same autoradiograph (Fig. 1 A, center) shows very low level expression of caveolin mRNA in brain and no detectable caveolin mRNA in spleen, kidney, liver and testis.

The Western blot analysis in Fig. 1 B shows that adipose tissue contains a larger amount of caveolin protein than any other tissue tested. Moderate levels of caveolin are seen in lung, skeletal muscle, and pancreas. Confirming the Northern blot analysis, little or no caveolin is detected in brain, spleen, kidney, liver, and testis. Interestingly, adipocytes have two forms of caveolin that are resolved by our SDS-PAGE conditions; all other tissues express only the slower migrating form under the conditions chosen. The ratio of the two forms in fat cells varies somewhat: in 3T3-L1 adipocytes the higher molecular weight form is much more abundant, whereas isolated adipocyte tissue from mouse (Fig. 1 B) or rat (see Fig. 8) fat pads display a significantly higher proportion of the lower molecular weight species.

Cultured 3T3-L1 fibroblasts offer a convenient system to study adipogenesis. Upon exposure to dexamethasone, isobutylmethylxanthine and insulin, these cells differentiate over a period of six to eight days from precursor fibroblasts into adipocytes that for most purposes are indistinguishable from adipocytes isolated from epididymal fat pads (4, 32). Caveolin mRNA is strongly induced between days 2 and 4 of adipocyte differentiation (Fig. 2 A), and exhibits a similar pattern of induction during differentiation as the mRNAs encoding the insulin receptor and GLUT4 (10), other adipocyte-specific products involved in insulin stimulation of glucose uptake. Relative to expression of the constitutive mRNA encoding hsp70, caveolin mRNA is induced 25-fold during the course of differentiation.

The Western blots in Fig. 2 B show that caveolin polypeptide is also induced over 20-fold during the eight days of adipocyte differentiation. A polyclonal antiserum against
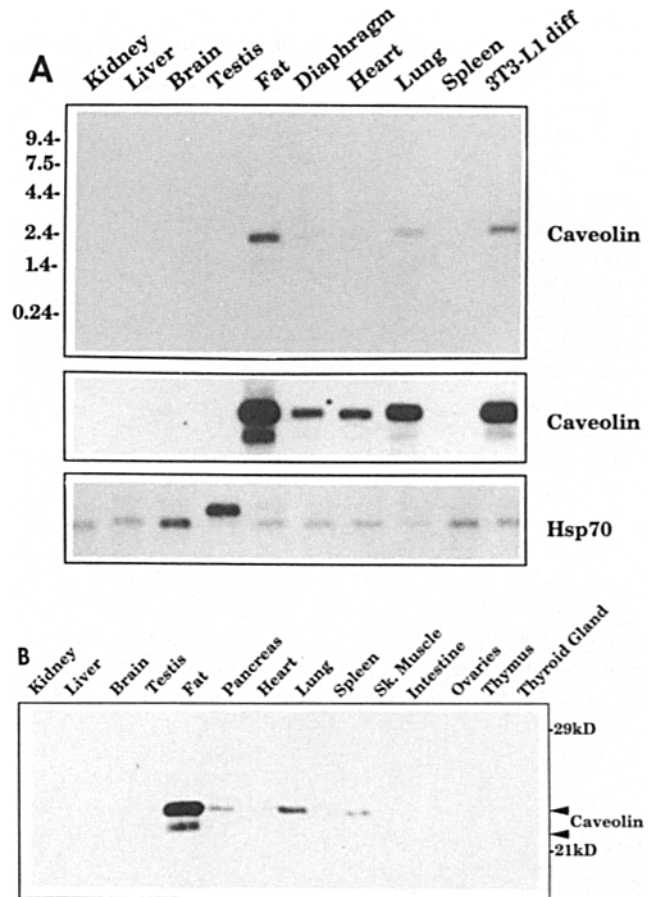

Figure 1. Caveolin mRNA and protein is most abundant in adipose tissue. (A) Northern blot. 0.5 μg of poly A–RNA harvested from different mouse tissues was separated on a 1% agarose gel containing formamide and transferred to a nylon membrane. The blots were probed with a fragment comprising the entire coding sequence of canine caveolin, stripped, and subsequently probed with a fragment comprising the coding sequence for mouse cytosolic hsp70 (25) to ensure equal loading. The top panel shows a Northern blot of caveolin, and the center panel a longer exposure of the same blot. The bottom panel shows the identical blot probed with hsp70. "3T3-L1 diff" indicates mRNA isolated from day 8 3T3-L1 adipocytes. Numbers on the left indicate molecular weight standards (in kb). (B) Western blot. Extracts of mouse tissues were prepared as described in Materials and Methods. From each tissue, 100 μg of protein was analyzed by SDS-PAGE. The gel was processed for Western blotting and analyzed with an antiserum against caveolin.

cytosolic hsp70 revealed (as a control for equal loading) equal amounts of the protein in 3T3 fibroblasts and adipocytes (Fig. 2 B, right) whereas the level of caveolin is increased 20-fold (left).

The Amount and the Protein Composition of the Caveolin-rich Fraction Increases during Adipogenesis

A stringent isolation procedure for caveolae takes advantage of the insolubility of caveolar components in Triton X-100 and the low buoyant density of caveolae due to their high content of glycosphingolipids (5, 30, 42). Thus, cells at different stages of adipocyte differentiation were solubilized in 1% Triton X-100, adjusted to equal amounts of protein, made 40% in sucrose, and overlaid with a linear 5 to 30% sucrose
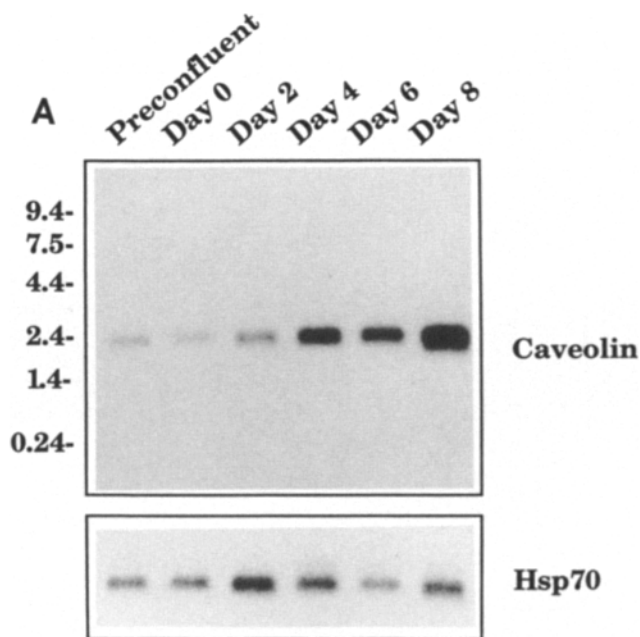
A

Figure 2. Caveolin mRNA and protein are induced during differentiation of 3T3-L1 fibroblasts to adipocytes. (*A*) Northern blot. 0.5 µg of poly A-RNA, harvested from cells at different days after induction of 3T3-L1 fibroblasts to adipocytes, was analyzed as described in the legend to Fig. 1 and probed sequentially with ^{32}P-labeled caveolin (*top*) and hsp70 DNAs (*bottom*). Numbers on the left indicate molecular weight standards (in kb). (*B*) Western blot. 50 µg of total cellular protein extracted from 3T3-L1 fibroblasts and 3T3-L1 adipocytes at day 8 of differentiation were resolved by SDS-PAGE, transferred to a nitrocellulose filter, and probed with antibodies to caveolin (*left*) and cytosolic hsp70 (*right*). The slightly changed mobility of hsp70 in adipocytes vs fibroblasts reflects the change in overall protein composition in that molecular weight range.

B

Figure 3. Protein composition of caveolin-rich domains during the course of adipocyte differentiation. As described in Materials and Methods, caveolae were harvested from 3T3 cells at different stages of adipocyte differentiation by extraction with Triton X-100 followed by equilibrium sucrose density gradient centrifugation. Prior to gradient centrifugation, the extracts were adjusted to contain the same amount of protein. The left part of *A* shows an SDS-PAGE analysis of the total protein isolated from preconfluent 3T3-L1 fibroblasts (*PC*) or cells at 0, 2, 4, and 6 d of differentiation, followed by silver-staining, showing that equal amounts of protein indeed were used. The right half of *A* shows a silver-stained SDS gel in which the total isolated caveolin-rich domains of these cells were analyzed. *B* shows a silver-stained SDS gel in which equal amounts of protein from the caveolin-rich domains of 3T3 fibroblasts (*Fb*) and 3T3 adipocytes at 8 d of differentiation (*Adi*) was analyzed. Numbers on the left of each panel indicate molecular weight standards (in kD).

gradient. After centrifugation for 16 h at 100,000 g, the caveolin-rich fraction can be visualized as a sharp band between 15–20% sucrose (30, 42). The center panel in Fig. 3 shows that the amount of caveolin-rich domains from cells at 6 d of differentiation is dramatically higher than that from preconfluent 3T3 fibroblasts. As evidenced by the silver-stained gels in the left panel of Fig. 3, the total extracts of cells at the different stages of differentiation contained approximately the same amount of protein. Caveolae isolated from cells at 8 d of differentiation contain a distinct and more complex pa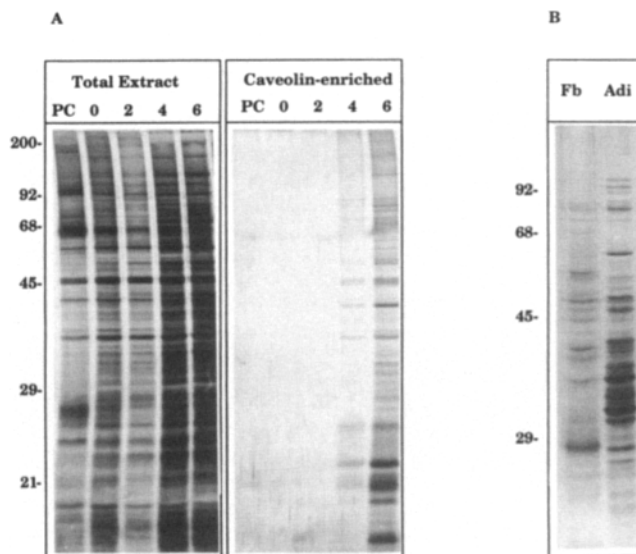ttern of proteins than do caveolae isolated from preadipocytes, as is demonstrated by the right panel of Fig. 3 where equal amounts of caveolar protein are analyzed, using a large amount of undifferentiated cells to obtain comparable levels of caveolin-rich domains.

Electron micrographs of the caveolae isolated from cells at 8 d of differentiation (Fig. 4) show that they are predominantly curved membrane fragments, a morphology similar to that of this fraction isolated from other types of cells (30, 42).

The experiment depicted in Fig. 5 shows that the caveolar fraction isolated from 3T3-L1 adipocytes is very pure. The first panel shows the GLUT4 distribution across the gradient. The second panel from the top shows that in adipocytes all of the caveolin, as expected, is recovered in the light-density caveolae-containing fractions 4 and 5. Importantly, proteins characteristic of the plasma membrane and several intracellular membranes are excluded from this low density fraction, demonstrating that this is a valid and specific procedure for isolation of adipocyte caveolin-rich membrane domains. Plasma membrane markers, such as the α_2-subunit of the Na^{+}/K^{+}-ATPase (52) as well as another previously characterized Triton-insoluble 45-kD protein (23) (not shown) are completely localized to the most dense fractions 8–12, which contain the bulk of the cellular membranes (third panel from the top). Similarly, the lysosome-associated membrane protein LAMP1, a glycosylated
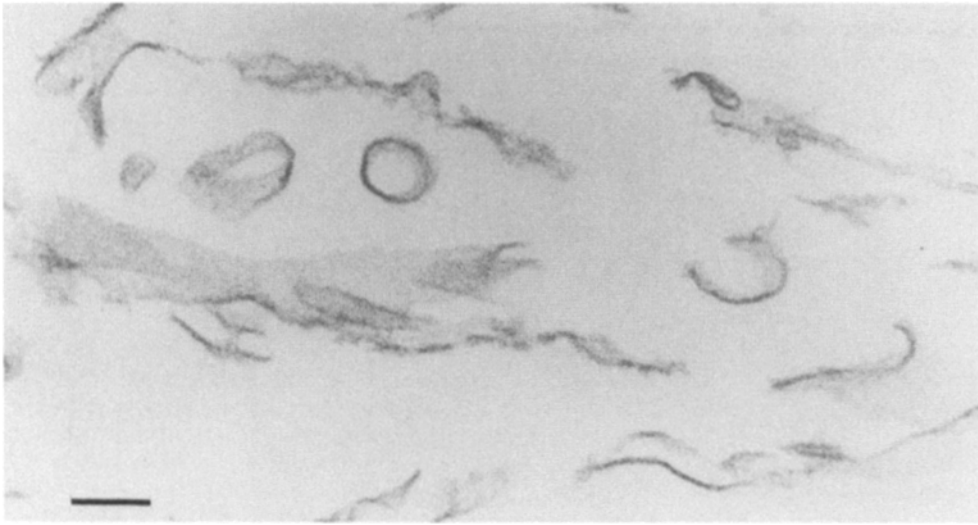
105–115-kD protein (11) is completely localized to fractions 8–12 (fourth panel from the top), as are several mitochondrial outer membrane proteins of 50 kD, 80 kD and 100 kD (fifth panel from the top) and one of 17-kD protein (sixth panel from the top) that are detected by one antiserum. No detectable amounts of these proteins are seen in fractions 4 and 5, which contain the adipocyte caveolin-rich membrane domains. Furthermore, >96% of total protein is excluded from these two fractions (bottom panel of Fig. 5). Additionally, the patterns of proteins in the caveolar fractions do not reflect the pattern of proteins in the total extracts (Fig. 3), thereby establishing that nonspecific aggregation of protein does not account for the composition of the caveolar fractions.

Caveolae May Be an Intermediate in Intracellular Trafficking of GLUT4

The experiments in the top panels of Figs. 5 and 6 show that, in 3T3-L1 adipocytes unstimulated by insulin, about 10–15% of total cellular GLUT4 is localized to the low density, caveolin-rich vesicular compartment that contains all of the caveolar marker caveolin (Fig. 5, panel 2). The remainder of the GLUT4 is localized to the bottom fractions of the gradient that contain the bulk of cellular membranes. Consistently, we find that 5 min after stimulation of 3T3-L1 adipocytes with insulin at 37°C, there is a twofold increase in GLUT4 associated with the caveolar fraction and a proportionate decrease in the amount of GLUT4 in fractions 8–12 that contain the bulk of the cellular membranes (Fig. 6, lower panel). This significant increase is transient, since at 20 min of insulin stimulation the amount of GLUT4 associated with caveolae has decreased to a level similar to that of unstimulated cells. The results of several independent repetitions of this experiment are tabulated in Table I.

Fig. 7 shows that a significant amount of caveolin is translocated from the low-density microsomal fraction to the plasma membrane after stimulation of 3T3-L1 adipocytes with insulin. On average, a 40% decrease of caveolin in the low-density microsomal fraction can be observed within 5 min after insulin stimulation. A significant fraction of GLUT4 is also translocated from the low-density microsomal fraction to the plasma membrane after insulin stimulation. The loss of caveolin from the low-density microsomal fraction increases with time of insulin treatment, and was reproduced in four independent experiments (Table II).

This experiment does not establish whether caveolin is in the same low-density microsomal vesicles as is the GLUT4 that is translocated to the plasma membrane in response to insulin, or whether it is present on other types of vesicles that undergo translocation in response to insulin. However, some intracellular vesicles that contain GLUT4 also contain caveolin, as is illustrated in Fig. 8. Rat adipocytes were used in these experiments, and we used a well-defined procedure for immunoisolating GLUT4-containing vesicles from the supernatant that remains after a 48,000 g centrifugation and that contains low-density microsomes and cytosol (7). GLUT4-containing vesicles were immunoisolated using polyclonal antibodies, directed against the cytoplasmic tail of GLUT4, that were immobilized on *Staphylococcus aureus* cells. These immunoisolated vesicles have been extensively characterized; a number of studies have shown that they contain transferrin receptors, insulin-like growth factor II receptors (50), and an unidentified 165-kD protein (33). Like GLUT4, these proteins are translocated to the plasma membrane after insulin stimulation. These immunoisolated vesicles are deficient in a number of proteins characteristic of other intracellular membranes; specifically, they are depleted in the Golgi and *trans*-Golgi reticulum enzymes galactosyltransferase and sialyltransferase (6). The vesicle preparation used in the experiment in Fig. 8 was the same one used in reference 33. After washing the beads, proteins were released in two steps: a first elution was performed using the non-ionic detergent $C_{12}E_8$ followed by a second and more stringent elution performed by boiling the immunoisolates in SDS. Fig. 8 (lane *1* and *3*) shows that GLUT4-containing vesicles also contain caveolin that is eluted predominantly in $C_{12}E_8$ and to a lesser extent in SDS; vesicles isolated using preimmune serum contain no caveolin (lanes 2 and 4). Under these conditions, ~10% of the caveolin in low-density microsomes is immunoisolated with GLUT4 vesicles.
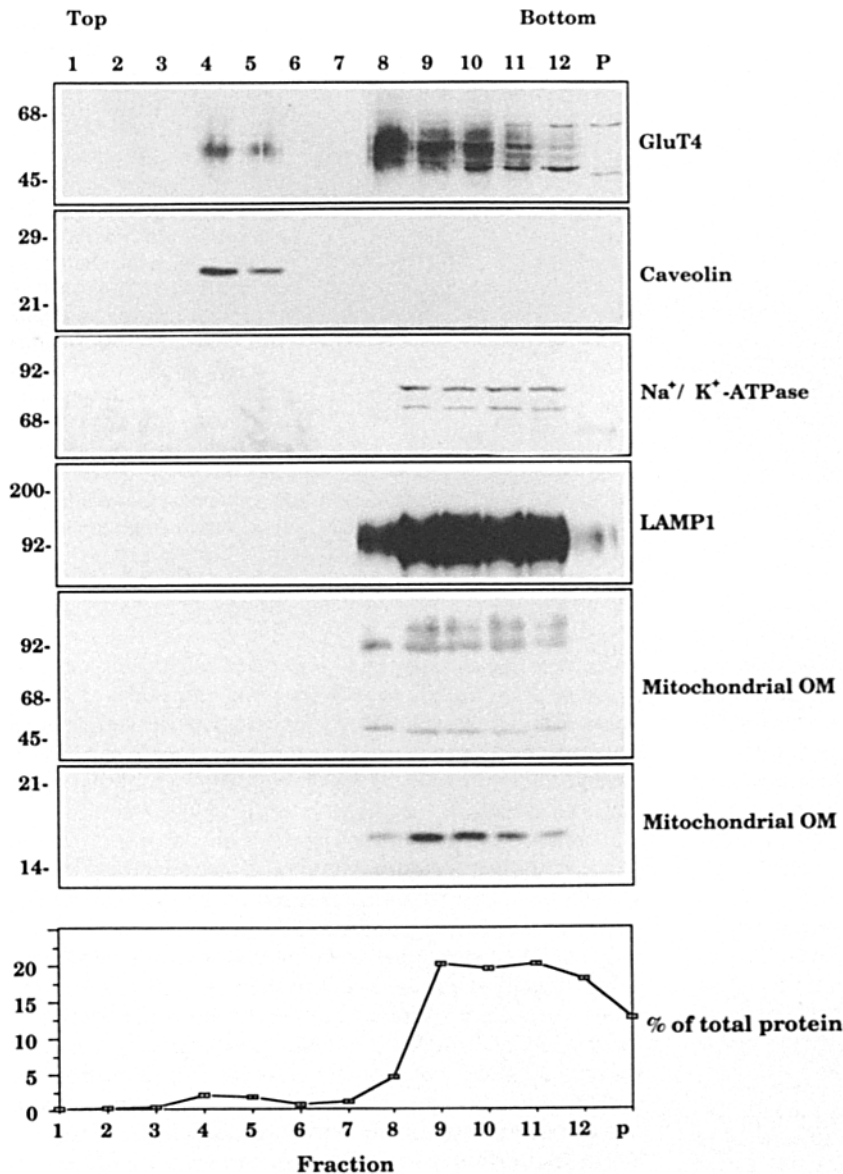
Figure 5. Subcellular localization of GLUT4 and caveolin in 3T3-L1 adipocytes. 3T3-L1 adipocytes at the eighth day of differentiation were lysed in buffer containing 1% Triton X-100, brought to 40% sucrose, and overlaid with a linear 5–30% sucrose gradient lacking detergent. After centrifugation for 16 h at 200,000 *g*, 12 fractions were harvested along with the pelleted material (*p*). An equal volume of each fraction was analyzed by SDS-PAGE and Western blotting for the distribution of (from top to bottom): GLUT4, caveolin, α_2-subunit of Na$^+$/K$^+$-ATPase, lysosome-associated membrane protein LAMP1, and an antiserum against purified rat mitochondrial outer membrane. Shown here are results obtained with the rabbit polyclonal antiserum against the α_2-subunit of Na$^+$/K$^+$-ATPase; identical results (not shown) were obtained with mouse monoclonal antibody McB2. The anti-outer membrane serum detected proteins of apparent molecular weight 40 to 150 kD and one protein of ∼17 kD; only these portions of the autoradiograms are shown. The overall distribution of protein across the gradient (*bottom*) was determined by the BCA protein assay (Pierce Chemical Co.), and the protein content of each fraction was expressed as a percentage of the total amount of protein recovered in the gradient.
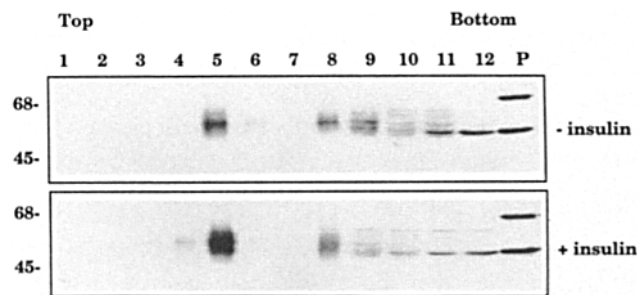

Figure 6. Translocation of GLUT4 into a caveolar fraction upon addition of insulin. 3T3-L1 adipocytes were serum-starved for 2 h and either left untreated or exposed for 6 min to 100 nM insulin at 37°C. Cells were immediately washed with ice-cold PBS and the caveolar fraction prepared as described above. Equal volumes from each fraction were analyzed by SDS-PAGE followed by Western-blotting with antibodies against GLUT4. Scanning of different exposures of the same gels revealed a twofold increase of GLUT4 in the caveolar fractions 4 and 5 with a corresponding decrease in the dense membrane fractions 8–12 after 5 min of insulin stimulation. The experiment shown corresponds to experiment 1 in Table I.

Table I. Change in the Amount of GLUT4 in the Caveolin-enriched Fraction after Addition of Insulin

Relative amount of GLUT4 in caveolae (fraction of total GLUT4)	0 min	5 min	20 min
Experiment 1	1.0	1.9	ND
	(0.23)	(0.44)	ND
Experiment 2	1.0	1.6	0.99
	(0.13)	(0.20)	(0.13)
Experiment 3	1.0	2.0	1.6
Experiment 4	1.0	1.2	1.0

Caveolin-enriched fractions were prepared before and after addition of 100 nM insulin to 3T3-L1 adipocytes for the indicated periods of time. As described in Materials and Methods and in the legend to Fig. 6, the low-density caveolin-rich fraction and the bulk of the cellular membranes were separated by equilibrium density gradient centrifugation. In Experiments *1* and *2*, the amount of GLUT4 in all fractions was quantified by Western blotting. The fraction of caveolar GLUT4 compared to total cellular GLUT4 was calculated at each time point and is indicated in parenthesis. The fraction of GLUT4 found in the caveolin-rich fraction before addition of insulin was set as 1.0. In the remaining experiments, only the caveolar fraction was analyzed and changes in GLUT4 content after insulin addition were normalized to the total amount of protein present in each sample before equilibrium density gradient centrifugation.
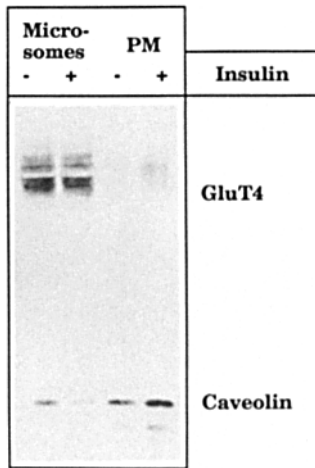
Micro-somes		PM		
-	+	-	+	Insulin

GluT4

Caveolin

Figure 7. Caveolin is translocated from low-density microsomes to the plasma membrane upon addition of insulin. 3T3-L1-adipocytes were exposed to 100 nM insulin for 6 min (+) or were left untreated (−). Microsomal and plasma membrane–rich fractions were isolated as detailed in Materials and Methods and as described in reference (3). These fractions were analyzed by SDS-PAGE followed by Western blotting with antibodies against GLUT4 and caveolin. Bound antibodies were visualized by enhanced chemiluminescence.

G4　NI　G4　NI

45-

29-

21-

$C_{12}E_8$　　SDS

Figure 8. GLUT4 vesicles also contain caveolin. As described in Materials and Methods, GLUT4-containing vesicles were immunopurified from a rat adipocyte low density microsomal preparation containing 22 μg of protein. For the immunopurification, either an antibody to the COOH terminus of GLUT4 (*G4*, lanes *1* and *3*) or a non-immune antiserum (*NI*, lanes *2* and *4*) was used. Elution of proteins bound to the immunobeads was done sequentially, first with the non-ionic detergent $C_{12}E_8$ (lanes *1* and *2*) followed by SDS (lanes *3* and *4*). Eluates were analyzed by SDS-PAGE and Western blotting with anti-caveolin antibodies. Numbers on the left indicate molecular weight markers.

Taken together, the experiments in Figs. 7 and 8 suggest that some low-density microsomes contain both GLUT4 and caveolin and are translocated to the plasma membrane in response to insulin stimulation. However, it is difficult to prove that the translocated caveolin and GLUT4 actually are in the same intracellular vesicles. About 10–20% of total cellular caveolin fractionates with the microsomal fractions. Table II indicates that upon prolonged exposure to insulin, greater than 50% of this microsomal caveolin has been translocated to fractions that cofractionate with the plasma membrane.

Caveolin Associates with a 32-kD Phosphoprotein after Exposure of Cells to Insulin

Caveolin was initially identified as a substrate for v-src, but is predominantly phosphorylated on serine residues in vivo (20, 42). We therefore tested the possibility that caveolin would be subject to phosphorylation in response to insulin. As judged by one-dimensional SDS-PAGE, caveolin is found in two major forms in adipocytes: a 22- and 26-kD form. Interestingly, only the 22-kD form is phosphorylated in vivo (Fig. 9 *A*, *left*) even though the 26-kD form is present in much higher abundance (Fig. 9 *A*, *right*). This suggests that the lower molecular weight form is not merely a degradation product, but is indeed a functional protein found in vivo. When isolated caveolae are incubated in vitro with γ-[^{32}P]-ATP, both forms of caveolin become radiolabeled (data not shown), suggesting that both forms of caveolin are potential substrates for a cellular protein kinase.

After insulin addition there is no change in the overall level of phosphorylation of caveolin (Fig. 9 *A*, *left*). By phosphoaminoacid analysis (not shown) we demonstrated that both in the absence and presence of insulin adipocyte caveolin is phosphorylated on serine residues; there is no phosphorylation of threonine or tyrosine residues. We do not
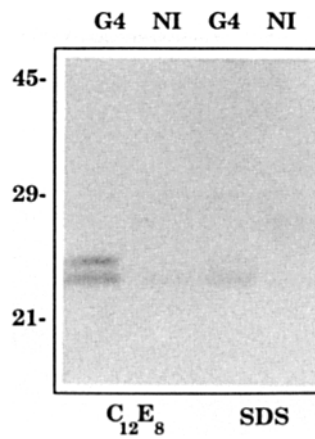
know the molecular difference(s) between the 22- or 26-kD forms of caveolin. Both species are subjected to multiple posttranslational modifications: isoelectric focusing followed by SDS-PAGE revealed that the upper band is split into three distinct species, while the lower molecular weight form gives rise to four species. This pattern is not affected by exposing cells to insulin (data not shown), and the nature of these modifications is currently under investigation.

Even though caveolin does not undergo a major change in phosphorylation upon exposure of cells to insulin, insulin stimulation leads to stable association of caveolin with a phosphorylated protein of 32 kD (Fig. 9 *B*). In this experiment 3T3-L1 adipocytes were metabolically labeled with [^{32}P]orthophosphate for 3 h, and then left untreated or exposed to insulin for 6 min. The overall pattern of phosphorylation of cellular proteins does not change after insulin addition (Fig. 9 *B*, *left*) while, after addition of insulin, a 32-kD phosphoprotein co-immunoprecipitates with caveolin under non-denaturing conditions (*right*). Since we are unable to detect this 32-kD protein by immunoprecipitation of cells labeled with [^{35}S]methionine and cysteine, we cannot determine whether or not the unphosphorylated form of this (unidentified) protein is associated with caveolin in the absence of insulin. However, this experiment does establish that after insulin addition there is a change in the pattern of phosphorylated proteins associated with caveolin.

Discussion

A principal result of this work is that caveolin mRNA and protein are induced greater than 20-fold during adipogenesis. This extends earlier morphological studies showing that

Table II. Change in the Amount of Caveolin in the Low-density Microsomal Fraction after Addition of Insulin

Time after insulin addition	0 min	2 min	5 min	20 min
Relative amount of caveolin in the low density microsomal fraction	1.00	0.70 ± 0.04	0.60 ± 0.14	0.40 ± 0.22

As described in Materials and Methods, plasma membrane, low-density microsomal, and other fractions were isolated from 3T3-L1 adipocytes before or after the addition of 100 nM insulin for the indicated periods of time. The amount of caveolin in all subcellular fractions was determined by Western blotting. The relative amount of caveolin found in the low-density microsomal fraction before the addition of insulin was set as 1.00 and in the different experiments corresponds from 10 to 20% of total cellular caveolin. Data were collected from four independent subfractionations; shown are the mean and standard deviation.
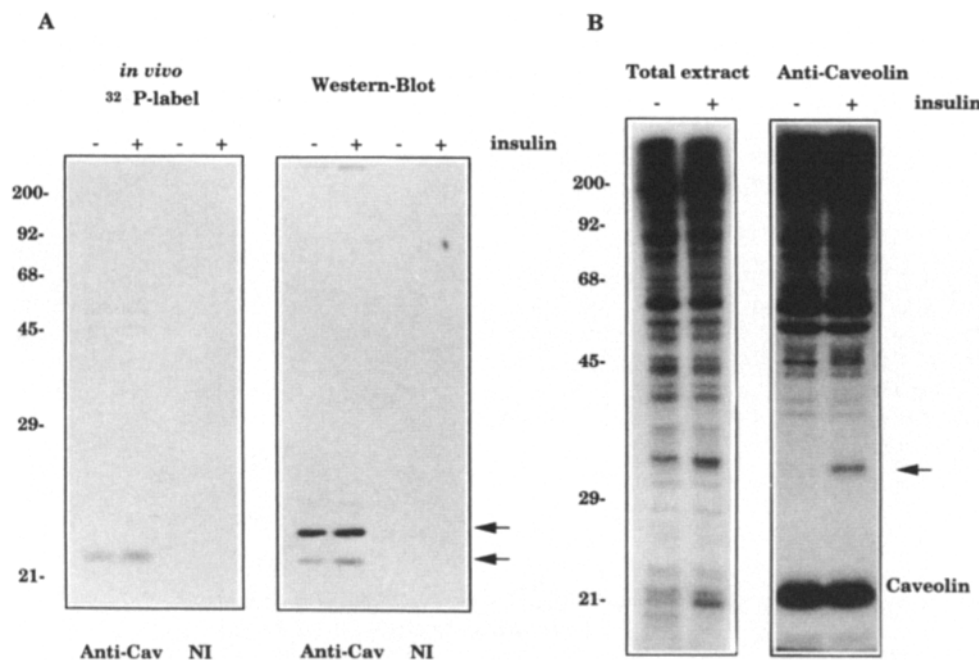
Figure 9. Caveolin is phosphorylated, and associates with a 32-kD phosphoprotein after exposure of cells to insulin. (*A*) The 22-kD but not the 26-kD form of caveolin is phosphorylated. 3T3-L1 adipocytes were metabolically labeled with [^{32}P]ortho-phosphate for 3 h and then left untreated (−) or exposed to 100 nM insulin for 6 min (+). Thereafter the cells were washed, lysed and immunoprecipitated with anti-caveolin (Anti-Cav) or an irrelevant serum (*NI*); Immunoprecipitates were analyzed by SDS-PAGE followed by transfer of the proteins to nitrocellulose. The filter was first subjected to autoradiography (*left*) and subsequently decorated with anti-caveolin antibodies to reveal all caveolin species present. Bound antibody was visualized with appropriate secondary antibody and enhanced chemiluminescence. (*B*) Association with a 32-kD phosphoprotein. 3T3-L1 adipocytes were metabolically labeled with [^{32}P]ortho-phosphate for 3 h and then left untreated (−) or exposed to 100 nM insulin for 6 min (+). All samples were subjected to SDS-PAGE and autoradiography. In the left panel 1% of the total cellular extract was analyzed. In the right panel, the remainder of the extracts were immunoprecipitated with anti-caveolin antibodies and analyzed. On the right the coprecipitating 32 kD protein is marked with an arrowhead and the caveolin band is labeled. Numbers on the left indicate the positions of molecular weight markers.

caveolae-like structures are abundant in adipocyte plasma membranes and that there is a ninefold increase in the number of these structures during differentiation of 3T3-L1 fibroblasts into adipocytes (16). Indeed, 3T3-L1 adipocytes, as well as fat tissue in situ, express an extremely high level of caveolin—higher than any other tissue. Induction of caveolin occurs primarily between days 2 and 4 of differentiation, the same time as induction of the insulin receptor and GLUT4. Our results suggest that caveolae might be involved in insulin-stimulated exocytosis of GLUT4 from intracellular vesicles to the plasma membrane, or in endocytosis of GLUT4 from the plasma membrane. We showed that in non-stimulated adipocytes about 10–15% of GLUT4 is in caveolae, and that immunoisolated vesicles that contain GLUT4 also contain caveolin. Upon addition of insulin to 3T3-L1 adipocytes there is a significant increase in the fraction of GLUT4 associated with caveolin-rich domains, a decrease in the amount of caveolin in low-density microsomes, and a corresponding increase in the amount of caveolin in the plasma membrane. Although in non-stimulated adipocytes the majority of caveolin fractionates with the plasma membrane while most GLUT4 associates with low-density microsomes, our results suggest that caveolae may be intermediates in the intracellular trafficking of GLUT4. From our results, we cannot conclude that plasma membrane GLUT4 resides in caveolae, and that caveolae are the actual site of glucose transport. Rather, the population of GLUT4 found in caveolae is likely to be a transient intermediate, on their way to or from the plasma membrane. Indeed, the amount of GLUT4 in caveolae increases significantly but transiently after stimulation with insulin and then returns to the basal level, whereas the amount of GLUT4 in the plasma membrane increases after insulin stimulation and remains at that elevated level as long as insulin is present.

Purity of Caveolae Isolated from 3T3-L1 Adipocytes

Our studies were greatly facilitated by a well characterized procedure for the biochemical isolation of caveolin-rich fractions (5, 30, 42). It takes advantage of the insolubility in Triton X-100 of caveolar components and of the low buoyant density of caveolae due to their high content of glycosphingolipids. The equilibrium sucrose gradients we used, combined with prior extraction in solutions of Triton X-100, allow effective separation of adipocyte caveolae from plasma membranes, from other cellular membranes, and from proteins associated with the cytoskeleton and nuclear matrix. A highly purified preparation of adipocyte caveolae can be harvested as a sharp band after ultracentrifugation. Attesting to its purity, this fraction completely lacks a number of proteins characteristic of the plasma membrane, lysosomal membrane, and mitochondrial membrane (Fig. 5). It also lacks a number of soluble cytosolic proteins we examined (data not shown). Similarly, both in MDCK cells and in lung tissue this fraction is greatly enriched in caveolin and is depleted of other proteins originating from intracellular membranes as well as from the plasma membrane (30, 42). Chang et al. (9) developed a quite different protocol for the purification of caveolae; the protein composition of their purified fractions is very similar to that of the caveolin-enriched membranes obtained using the flotation assay described in (30, 42) and used in this paper.

During the course of adipocyte differentiation there is a large increase in the amount of caveolae, as well as an increase in the complexity of their protein composition. This

suggests that caveolae play an important role in adipocyte physiology, possibly in the insulin regulation of GLUT4 translocation. Other roles for caveolae cannot be excluded. For instance, they may be the sites of transport of fatty acids into the adipocyte (Trigatti, B. L., R. G. W. Anderson, and G. E. Gerber, manuscript submitted for publication).

Intracellular Trafficking of GLUT4 in Adipocytes

By cell fractionation techniques, glucose transporters localize to the low-density microsomal compartment (LDM) of unstimulated rat adipocytes and ~30–50% are translocated to the plasma membrane after insulin addition (45). In 3T3-L1 cells, a lower but still significant fraction of intracellular GLUT4 is translocated to the plasma membrane in response to insulin. Our ultrastructural studies of unstimulated adipocytes isolated from rat epididymal fat pads (48) indicated that GLUT4 was predominantly associated with smooth, non-clathrin–coated plasma membrane invaginations, surface-connected vesicles, or vesicles that were within 75 nm of the plasma membrane. These structures are reminiscent of caveolae, but no effort was made to determined if they contained caveolin. As judged by immunogold labeling, treatment with insulin resulted in an ~20-fold increase in plasma membrane GLUT4, to 52% of total GLUT4, and a proportional decrease in intracellular transporters. This effect was reversed when the insulin-stimulated cells were washed and reincubated in insulin-free media before labeling. Using similar techniques, Slot and colleagues found in rat brown adipose tissue an even greater insulin-stimulated translocation of GLUT4 (47). They also reported similar effects of insulin on GLUT4 localization in cardiac muscle cells (46). Thus, the main effect of insulin treatment is to cause translocation of GLUT4 transporters to the plasma membrane; this involves flow of transporters from invaginations to the cell surface and fusion of sub-plasma membrane vesicles with the plasma membrane.

By labeling rat adipocyte cell surface glucose transporters with an impermeable, photoreactive glucose analogue, Jhun et al. (27) concluded that GLUT4 constantly recycles between the cell surface and intracellular membranes. Insulin increased the rate of GLUT4 exocytosis 3.3-fold and inhibited the rate of GLUT4 endocytosis 2.8-fold. Experiments in which cell surface GLUT4 was detected by sensitivity to trypsin also indicated that insulin inhibited the rate of GLUT4 endocytosis (13). Using a different impermeable, photoreactive glucose analogue, however, others showed that insulin had no effect on GLUT4 or GLUT1 endocytosis, and increased exocytosis of both transporters (43, 56). The entire cellular complement of GLUT4 recycles. Importantly, following insulin stimulation of adipose cells in the basal state, the appearance of GLUT4 in the plasma membrane preceded the increase in glucose transport activity, suggesting that there might be some activation of transport by plasma membrane GLUT4 (13, 43, 56). Nonetheless, translocation of GLUT4 to the plasma membrane is the primary mechanism of activation of glucose transport induced by insulin.

Caveolin and Insulin-stimulated Redistribution of GLUT4

Caveolae could be an intermediate in insulin-stimulated exocytosis of GLUT4 to the plasma membrane. A mathematical analysis of GLUT4 trafficking showed that there must be at least two intracellular pools of GLUT4 in order to account for the observed kinetics of insulin-stimulated glucose transport (24). One of these intracellular pools could contain caveolin. In 3T3-L1 adipocytes over half of the caveolin found in the low-density microsomal compartment is translocated to the plasma membrane in response to insulin stimulation (Fig. 7 and Table II), a larger fraction than of intracellular GLUT4. Thus, caveolin could also be found on several subpopulations of caveolae within the cell, including one that contains GLUT4, all of which are translocated to the plasma membrane in response to insulin. Fujimoto and colleagues obtained preliminary evidence for different subpopulations of caveolae in a number of different cell types (18, 19). In adipocytes, several membrane proteins translocate to the plasma membrane in response to insulin (12, 14, 54). For instance, after insulin stimulation there are increased amounts of the transferrin receptor on the plasma membrane, even though the majority of transferrin receptor does not colocalize to the intracellular vesicles that contain GLUT4 (50). This indicates that there may be different intracellular vesicles that are translocated to the plasma membrane in response to insulin, and caveolin could be associated with any of these.

Alternatively, caveolae could be intermediates in endocytosis of some, if not all, GLUT4 from the plasma membrane. Glucose transporters could be recruited into the caveolae and be transferred to an endosomal compartment for recycling. If insulin only stimulated exocytosis of GLUT4, and not the rate of endocytosis, the higher amount of plasma membrane GLUT4 after insulin addition would lead to an increase in the amount of GLUT4 in early endosomal vesicles; this could be the explanation for the increase in GLUT4 localized to caveolae after insulin addition. Arguing against this possibility is our finding that the association of GLUT4 with caveolae is transient, while as long as insulin is present the amount of plasma membrane GLUT4 remains at a constant, high level, about 50% of cellular GLUT4. Thus the fraction of GLUT4 in early endosomes should be increased as long as insulin is present.

In unstimulated adipocytes much GLUT4 is localized to invaginations of the plasma membrane or to vesicles connected to the cell surface membrane, structures similar to caveolae (48). Upon homogenization, these invaginations could be sheared from the plasma membrane and form small vesicles that sediment in the low-density microsomal fraction. This could explain the subfraction of low-density microsomes we detected that contain both GLUT4 and caveolin, though it does not establish whether these are involved in endo- or exocytosis of GLUT4. Robinson et al., studying fragments of the plasma membrane, localized some GLUT4 to coated pits (39). Using immuno-electron microscopy, they could not detect GLUT4 in caveolae attached to the plasma membrane. However, the particular GLUT4 epitope might be masked in caveolae (48) and therefore detected only upon biochemical isolation of caveolae. Alternatively, Robinson et al. (39) used different techniques for sample preparation than did Smith (48); the freeze-etch/rotary shadowing procedure used by Robinson et al. might not be optimal for visualization of caveolar GLUT4.

Caveolae and Signal Transduction by the Insulin Receptor

Jarett and co-workers (22) showed that in adipocytes the in-

sulin receptor is predominantly localized to plasmalemmal micropinocytotic invaginations (caveolae) that occupied a significant percentage (~13%) of the total plasma membrane. In contrast, coated pits (a more conventional route for endocytosis of receptors) occupied about 0.4% of the plasma membrane, only about one fifth of the density seen for coated pits in fibroblasts. While in other cell types the insulin receptor is internalized through coated pits, in adipocytes caveolae may preferentially be used for this purpose. The presence of the insulin receptor in caveolae together with other key signal transduction proteins (30, 42) could concentrate many molecules required for insulin-triggered translocation of GLUT4.

Association of Caveolin with a 32-kD Phosphoprotein after Insulin Addition

Adipocytes contain two forms of caveolin (a major species of 26-kD and a minor 22-kD form). Occasionally, upon mild denaturation, we could detect hetero- and homodimers of these two forms of caveolin, as judged by the appearance of SDS–resistant dimers upon SDS-PAGE and immunoblotting with anti-caveolin antibodies (data not shown). We do not yet know whether these dimers reflect a functional interaction in vivo or are merely a solubilization artifact.

Caveolin is subject to a variety of posttranslational modifications. Phosphorylation is one, since caveolin was originally identified as a major substrate for v-src. In adipocytes in vivo, only the low molecular weight form is phosphorylated, whereas our unpublished data showed that both the 22- and 26-kD form can be phosphorylated in vitro in cell extracts. This suggests that phosphorylation alone cannot account for the difference in molecular weight of the two major caveolin species. However, caveolin is subject to a number of other posttranslational modifications, suggestive of a highly regulated protein. Two-dimensional gel electrophoresis (not shown) reveals the existence of at least seven distinctive forms, possibly reflecting not only differences in phosphorylation but also in other modifications.

Given the localization of the insulin receptor to caveolae, it is possible that caveolin is a substrate for the insulin receptor. In several experiments, we did not detect a significant increase of either tyrosine or serine/threonine phosphorylation of caveolin after insulin addition. However, we did identify a 32-kD phosphorylated protein that is associated with caveolin upon addition of insulin. The identity of this protein is unknown. It also remains to be determined whether the unphosphorylated form of the protein is associated with caveolin in the absence of insulin and is phosphorylated after insulin addition, or whether insulin addition leads to association of this protein with caveolin. Nor do we know whether this 32-kD protein is a direct substrate for the insulin receptor or whether it is phosphorylated by another kinase that in turn is activated by the insulin receptor. The identification of this protein will provide important clues as to the role of caveolin in the cellular response to insulin.

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