Expression and Compartmentalization of Caveolin in Adipose Cells: Coordinate Regulation with and Structural Segregation from GLUT4

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Abstract. Native rat adipocytes and the mouse adipocyte cell line, 3T3-L1, possess transport vesicles of apparently uniform composition and size which translocate the tissue-specific glucose transporter isoform, GLUT4, from an intracellular pool to the cell surface in an insulin-sensitive fashion. Caveolin, the presumed structural protein of caveolae, has also been proposed to function in vesicular transport. Thus, we studied the expression and subcellular distribution of caveolin in adipocytes. We found that rat fat cells express the highest level of caveolin protein of any tissue studied, and caveolin is also expressed at high levels in cardiac muscle, another tissue possessing insulin responsive GLUT4 translocation. Both proteins are absent from 3T3-L1 fibroblasts and undergo a dramatic coordinate increase in expression upon differentiation of these cells into adipocytes. However, unlike GLUT4 in rat adipocytes not exposed to insulin, the majority of caveolin is present in the plasma membrane. In native rat adipocytes, intracellular GLUT4 and caveolin reside in vesicles practically indistinguishable by their size and buoyant density in sucrose gradients, and both proteins show insulin-dependent translocation to the cell surface. However, by immunoadsorption of GLUT4-containing vesicles with anti-GLUT4 antibody, we show that these vesicles have no detectable caveolin, and therefore, this protein is present in a distinct vesicle population. Thus, caveolin has no direct structural relation to the organization of the intracellular glucose transporting machinery in fat cells.

CAVEOLIN is a membrane protein originally discovered as a major substrate of *src* kinase in Rous sarcoma virus–transformed chick embryo fibroblasts (Glenney and Zokas, 1989; Glenney, 1989). In 1992, it was revealed to be a constituent of the *trans*-Golgi network (TGN) and TGN-derived transport vesicles (Kurzchalia et al., 1992), and of caveolae, small (50-80 nm) invaginations of the cell surface which are postulated to mediate the intracellular transport of small molecules (Rothberg et al., 1992; Dupree et al., 1993). Protein complexes containing caveolin, presumed to be representative of intact caveolae, that have been isolated on the basis of their Triton X-100 insolubility have also been shown to contain many receptors and associated signal-transducing proteins (for review see van Deurs et al., 1993; Anderson, 1993; Kurzchalia et al., 1994). Molecular cloning of caveolin demonstrated that it consists of 178 amino acids with one hydrophobic region (33–40 amino acids) close to the COOH terminus (Kurzchalia et al., 1992; Glenney and Soppet, 1992). Its membrane topology is not yet understood however, since there may be more than one membrane-spanning domain in the hydrophobic region of caveolin (Dupree et al., 1993), and there is also evidence that caveolin may be present as an oligomeric complex (Glenney, 1989; Dupree et al., 1993). After homogenization of epithelial cells, a substantial part of the total caveolin is found in detergent-insoluble complexes with different proteins, many of which have glycosyl-phosphatidylinositol anchor (Kurzchalia et al., 1992; Sargiacomo et al., 1993; Lisanti et al., 1993). Although the direct role of caveolin in compartmentalization of GPI-linked proteins and even in the organization of cell surface caveolae is far from being clear (Mayor et al., 1994; Smart et al., 1994; Fra et al., 1994), this protein is still very likely to be involved in some aspect of intracellular vesicular transport, and/or cell surface organization.

Thus, we decided to examine if caveolin may play such a role in adipose cells which possess a well-characterized system of vesicle-mediated protein transport of great physiological importance, namely the insulin-dependent translocation of the fat/muscle specific glucose transport, GLUT4, from an intracellular, vesicular storage pool to the plasma membrane (for review see Birnbaum, 1992; James and Piper, 1994; Mueckler, 1994; Stephens and Pilch, 1995). In fat cells, this translocation results in a 10–20-fold increase in the number of cell surface GLUT4 molecules (Zorzano et al., 1989; Holman et al., 1990). It is mainly or exclusively by this mechanism that postprandial glucose homeostasis is maintained in mammalian organism, primarily as a result of glucose uptake into muscle (Kraegen et al., 1985). However,

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most of what we know about this translocation process derives from the study of fat cells because of their comparative ease of experimental manipulation, particularly, a relatively precise subcellular fractionation scheme (Simpson and Cushman, 1986).

The insulin-dependent regulation of GLUT4 vesicular cycling raises a number of questions of general interest in the area of membrane protein trafficking and targeting. Among these are whether GLUT4 trafficking occurs via a normal endosomal pathway such as that of receptor-mediated endocytosis, or if it occurs via specialized vesicles such as those used by neuronal or endocrine cells for the secretion of neurotransmitters and hormones, respectively. A related question is whether the composition of GLUT4 vesicles is unique, and if so, what are the structural proteins involved in the organization of the glucose transporting machinery in insulin-sensitive cells. In light of the similarity in the tissue distribution of GLUT4 and caveolin, and their apparently identical regulation in cultured adipocytes in response to differentiation and to tumor necrosis factor treatment, we studied the subcellular distribution of caveolin in rat adipocytes in the basal and insulin-stimulated states. In resting fat cells, the majority of caveolin (ca. 70%) is localized to the plasma membrane, whereas the distribution of GLUT4 is exactly the opposite, ≥90% intracellular. Both proteins translocate to the plasma membrane in response to insulin, but from entirely different vesicular populations. These observations, together with morphological data from the other laboratories showing the exclusion of GLUT4 from plasma membrane caveolae (Voldstedlund et al., 1993; Slot et al., 1991*a*,*b*), argues against the direct involvement of caveolin in the formation of the glucose transporting machinery in adipocytes.

Materials and Methods

Antibodies

In the present work, we have used a monoclonal anti-GLUT4 antibody, 1F8 (James et al., 1988), and anti-caveolin mono- and polyclonal antibodies purchased from Transduction Laboratories (Lexington, KY).

Culture and Differentiation of 3T3-L1 Cells

Murine 3T3-L1 adipocytes were cultured, maintained, and differentiated as described previously (Stephens and Pekala, 1991; Cornelius et al., 1988).

Adipocyte Fractionation

Adipocytes were isolated from the epididymal fat pads of male Sprague Dawley rats (150–175 g) by collagenase digestion (Rodbell, 1964) and transferred to KRP buffer (12.5 mM Hepes, 120 mM NaCl, 6 mM KCl, 1.2 mM $MgSO_4$, 1 mM $CaCl_2$, 0.6 mM Na_2HPO_4, 0.4 mM NaH_2PO_4, 2.5 mM D-glucose, 2% BSA, pH 7.4) heated to 37°C. Insulin was administered to cells (where indicated) to final concentration of 10 nM for 15 min, and then 0.2 M KCN was added to a final concentration of 2 mM for 5 min. Cells were washed three times with HES buffer (20 mM Hepes, 250 mM sucrose, 1 mM EDTA, 5 mM benzamidine, 1 mM phenylmethanesulfonyl fluoride (PMSF), 1 μM pepstatin, 1 μM aprotinin, 1 μM leupeptin, pH 7.4) at 18°C, homogenized with a Potter-Elvehjem Teflon pestle, and subcellular fractions were prepared by differential centrifugation as previously described (Simpson et al., 1983). 3T3-L1 adipocytes were homogenized and fractionated according to the same protocol. For further fractionation by velocity or density gradient centrifugation, the light microsome (LM)[1] fraction was pelleted onto a 50% sucrose cushion (to avoid a resuspension step) and transferred to PBS (containing standard cocktail of protease inhibitors) by passing through a 5-ml Presto Column (Pierce, Rockford, IL).

Isolation of Crude Membrane Fractions from Different Tissues

Fat, heart, kidney, pancreas, lung, and spleen were obtained from 1–3 male rats, washed three times with 5 ml of cold HES buffer and homogenized in a Polytron for 20 s. Homogenates were spun for 10 min at 5,000 rpm, and the supernatant was then centrifuged at 48,000 rpm for 1.5 h onto a 50% sucrose cushion in a Ti-70.1 rotor. Pellets were collected from the cushion and resuspended in PBS with protease inhibitors.

Fractionation of Intracellular Microsomes in Sucrose Gradients

Light microsomes (0.1–0.2 ml, 1–2 mg/ml protein) from rat adipocytes were suspended in PBS and loaded onto a 4.6-ml continuous sucrose gradient prepared in PBS (10–30% sucrose for velocity centrifugation, and 10–50% sucrose for density gradient centrifugation). The former were centrifuged for 55 min, the latter for 18 h in a SW-50.1 rotor at 48,000 rpm, and each was fractionated into 26 fractions starting from the bottom of the tube. For Triton X-100 solubilization, the LM were treated with 1% Triton X-100 for 3 h at 4°C, spun for 15 min in a Microfuge and the supernatant was centrifuged in a 5–20% sucrose gradient prepared on PBS with 0.1% Triton X-100 for 16 h at 42,000 rpm in a SW-50.1 rotor. Gradients were separated into 27 fractions.

Immunoadsorption of GLUT4 Vesicles

Protein A purified 1F8 antibody, as well as nonspecific mouse IgG (Sigma Chem. Co., St. Louis, MO), were each coupled to acrylic beads (Reacti-gel GF 2000; Pierce) at a concentration 0.8–1.1 mg antibody per ml of resin according to manufacturer's instructions. Before usage, the beads were saturated with 2% BSA in PBS for at least 1 h and washed with PBS. The LM from rat adipocytes were incubated separately with each of the specific and nonspecific antibody-coupled beads overnight at 4°C. The beads were washed five times with PBS and the adsorbed material was eluted with Laemmli's (1970) sample buffer without 2-mercaptoethanol.

Immunoprecipitation of Caveolin and GLUT4 from Triton-solubilized Microsomes

15 μg of polyclonal anti-caveolin antibodies (Transduction Laboratories), or the same amount of nonspecific rabbit IgG was cross-linked to 0.2 ml of protein A–Trisacryl (Pierce) with dimethylpimelimidate (Harlow and Lane, 1988). LM were solubilized with 1% Triton X-100 (final concentration) for 2–3 h at 4°C and incubated with the beads overnight at 4°C. The beads were washed five times with PBS with 1% Triton X-100, 10 mM Tris (pH 7.4), and the adsorbed material was eluted with Laemmli's (1970) sample buffer without 2-mercaptoethanol. For GLUT4 immunoprecipitation, 1F8-beads were used instead of α-caveolin beads under the same experimental conditions.

Gel Electrophoresis and Immunoblotting

Proteins were separated in SDS-polyacrylamide (acrylamide from National Diagnostics) gels according to Laemmli (1970) and transferred to Immobilon-P membrane (Millipore, Bedford, MA) in 25 mM Tris, 192 mM glycine. After transfer, the membrane was blocked with 10% nonfat dry milk in PBS for 2 h at 37°C. GLUT4 and caveolin were visualized by sequential treatment with specific antibodies, HRP-conjugated secondary antibodies and an enhanced chemiluminescent (ECL) substrate kit (NEN).

Quantification of Proteins after Western Blotting

Where ^{125}I-labeled secondary antibodies were used, after autoradiography, bands were cut from the membrane and counted in a γ-counter. When HRP-conjugated secondary antibodies were used, the autoradiograph was scanned in a computing densitometer (Molecular Dynamics, Sunnyvale, CA). For some experiments both approaches were used with virtually identical results. Protein content was determined with BCA kit (Pierce) according to manufacturer's instructions.

1. *Abbreviations used in this paper*: HM, heavy microsome; LM, light microsome; PM, plasma membrane.

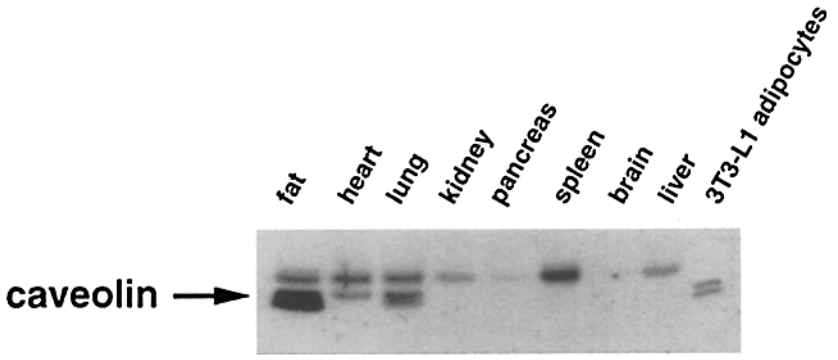

Figure 1. Tissue distribution of caveolin in rat. A total, postnuclear membrane fraction was isolated from the tissues indicated as described in Materials and Methods. Membrane protein (40 μg each) was electrophoresed, transferred to PVDF filter, and blotted with an anti-caveolin antibody as described in Materials and Methods. The caveolin was visualized by chemiluminescence.

Results

The tissue specific expression of caveolin is demonstrated by Western blot analysis in Fig. 1. The level of caveolin expression varies for different tissues, with the greatest expression level being observed in adipocytes. Heart, lung, and 3T3-L1 adipocytes also have appreciable levels of caveolin, although significantly less than in native fat cells, while other tissues do not express caveolin at detectable levels at an exposure of the gel that gives a strong signal for adipocytes. The band seen in the lane for spleen membranes (and elsewhere) is a nonspecific contaminant of lower electrophoretic mobility.

Given that the two insulin-sensitive tissues of fat and cardiac muscle express caveolin along with GLUT4, we decided to compare expression pattern of both proteins under conditions where GLUT4 expression is strongly regulated. For this purpose, we took advantage of the well-characterized process of in vitro differentiation of 3T3-L1 cells from fibroblasts to adipocytes. These cells are a useful model for studying many aspects of fat cell biology in vitro, as they have been shown to have essentially all the morphological and biochemical characteristics of native adipocytes (Green and Kehinde, 1976, 1979). Fig. 2 demonstrates that little or no expression of caveolin and GLUT4 is seen in the 3T3-L1 preadipocytes and both proteins are markedly induced upon cellular differentiation. Moreover, whereas most of the GLUT4 is in the LM, an internal membrane fraction of these cells, caveolin is found mostly in the plasma membrane (PM).

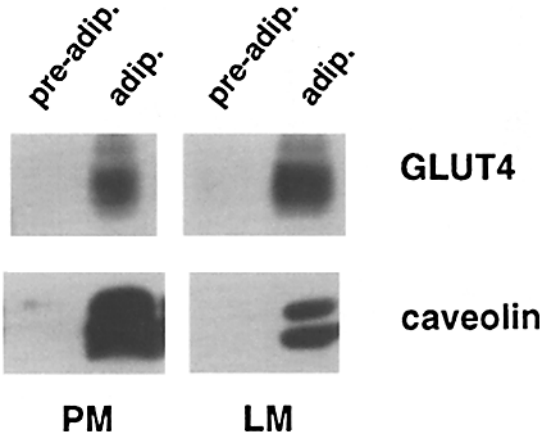

Figure 2. Caveolin and GLUT4 are induced during differentiation of 3T3-L1 fibroblasts into adipocytes. 3T3-L1 fibroblasts (lanes *1* and *3*) were differentiated into adipocytes (lanes *2* and *4*) in vitro as described in Materials and Methods. Cells were homogenized and fractionated into plasma membrane (*PM*) and intracellular light microsomes (*LM*). These fractions (50 μg of each) were electrophoresed and analyzed by Western blotting with anti-caveolin and anti-GLUT4 (1F8) antibodies.

For further examination of the intracellular localization of caveolin and its possible involvement in the organization of the insulin-sensitive glucose transporter machinery, we switched to native rat adipocytes because, as previously mentioned, they are exceptionally well suited to subcellular fractionation and have been very well characterized with regard to insulin-stimulated glucose transport (Simpson and Cushman, 1986). Thus, rat adipocytes were treated or not treated with insulin and were fractionated into subcellular fractions by differential centrifugation according to Simpson et al. (1983), and the aliquots of each fraction were electrophoresed. Coomassie staining of the gel does not reveal any noticeable changes in total polypeptide composition of any subcellular fraction after insulin treatment, and further more, cellular insulin exposure causes no changes in the subcellular distribution of appropriate marker enzymes (Simpson et al., 1983; and data not shown). Western blotting with anti-caveolin monoclonal antibodies (Fig. 3) demonstrated that most of the caveolin, as expected, is found in the plasma membrane. The heavy and light microsomes (HM and LM), which are enriched in ER and Golgi, respectively, according to the marker enzymes distribution (Simpson et al., 1983), as well as the combined mitochondria and nuclei fraction (M/N) also contains some caveolin (in this last case of M/N,

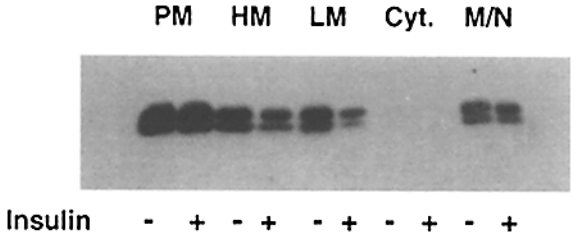

Figure 3. Subcellular distribution of caveolin in rat adipocytes treated and not treated with insulin. Membrane fractions (10 μg of total protein each), obtained as described in Materials and Methods from insulin-treated or -untreated rat adipocytes, were electrophoresed in a 10% polyacrylamide gel and analyzed by Western blotting using HRP-conjugated secondary antibodies and a chemiluminescence substrate kit. *PM*, plasma membranes; *HM*, heavy microsomes; *LM*, light microsomes; *Cyt.*, cytosol; *M/N*, mitochondria and nuclei.

almost certainly due to plasma membrane contamination). The cytosol fraction is completely devoid of this protein showing that there is virtually no free soluble caveolin in adipocytes, whereas it is completely incorporated into membrane or membrane-associated subcellular structures.

Quantitative analysis of the results of subcellular fractionation by computer densitometry of Western blots shows that 50% of total caveolin from the basal adipocytes and 70% of the caveolin from insulin-treated cells can be recovered in the plasma membrane fraction, and the combined fraction of mitochondria and nuclei contains 15–20% of total caveolin, presumably of plasma membrane origin. Intracellular caveolin shows a marked insulin-dependent translocation, most probably to the cell surface (see below), and decreases from 13–6% of total caveolin in the HM and from 15–5% in the LM fraction.

To further characterize the nature of caveolin-containing intracellular microsomes, total LM fractions from insulin-treated and -untreated adipocytes were fractionated in sucrose velocity gradients (Fig. 4). The upper panel of this figure shows the distribution of total LM protein which is essentially the same in the absence and in the presence of insulin (data not shown, Kandror et al., 1995). Caveolin-containing vesicles were found to have a specific sedimentational distribution very different from that of the total LM, peaking in fractions 9–15 where there is very little total protein. This picture is very similar to the sedimentational behavior of GLUT4-containing vesicles from adipocytes (Fig. 4), although caveolin has a more widespread distributional pattern. Also, and as previously shown for GLUT4 vesicles (Kandror et al., 1995), there is a shift towards a higher sedimentation value for vesicles isolated from insulin-treated adipocytes and the substantial overlapping of caveolin- and GLUT4-containing vesicles is maintained (*bottom panel*, Fig. 4). The signal from the insulin-treated adipocytes is diminished because ca. 50% of the GLUT4 vesicles (Zorzano et al., 1989) and presumably the caveolin-containing vesicles as well (Fig. 3) have moved to and fused with the plasma membrane.

To determine the buoyant density of caveolin-containing structures, we centrifuged light microsomal membranes to equilibrium in a sucrose density gradient (Fig. 5). Under these conditions, we also see almost complete overlapping of GLUT4 and caveolin-containing vesicles, both having a buoyant density in sucrose solution of 1.12–1.14 gm/cm³.

As shown in Figs. 3–5, the amount of intracellular cav-

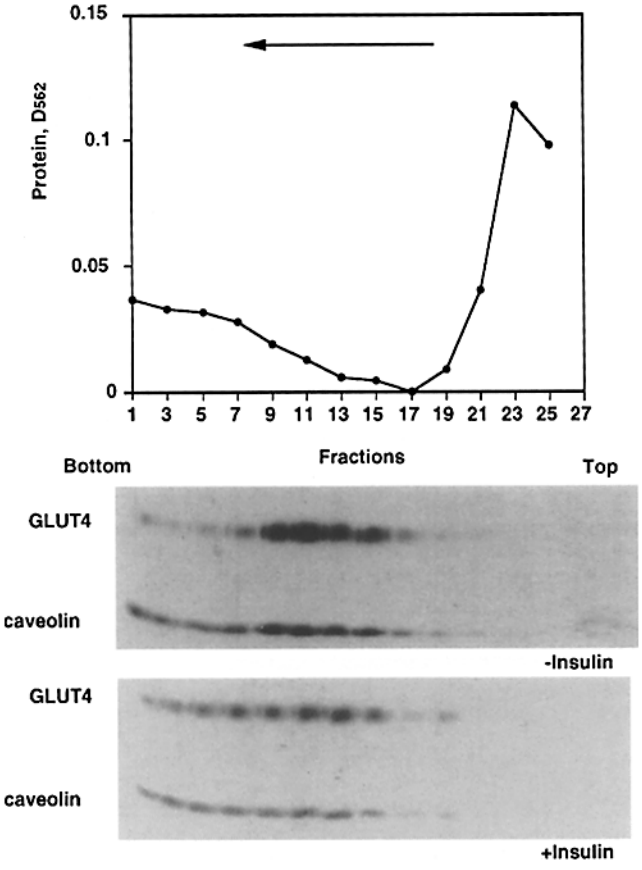

Figure 4. Caveolin- and GLUT4-containing vesicles have similar sedimentational distribution in sucrose gradients. LM (0.2 mg protein) from insulin-treated and -untreated adipocytes was fractionated in a 10–30% sucrose gradient as described in Materials and Methods. The horizontal arrow shows the direction of sedimentation. After centrifugation, odd fractions were collected and analyzed for caveolin and GLUT4 by Western blotting as in Figs. 2 and 3.

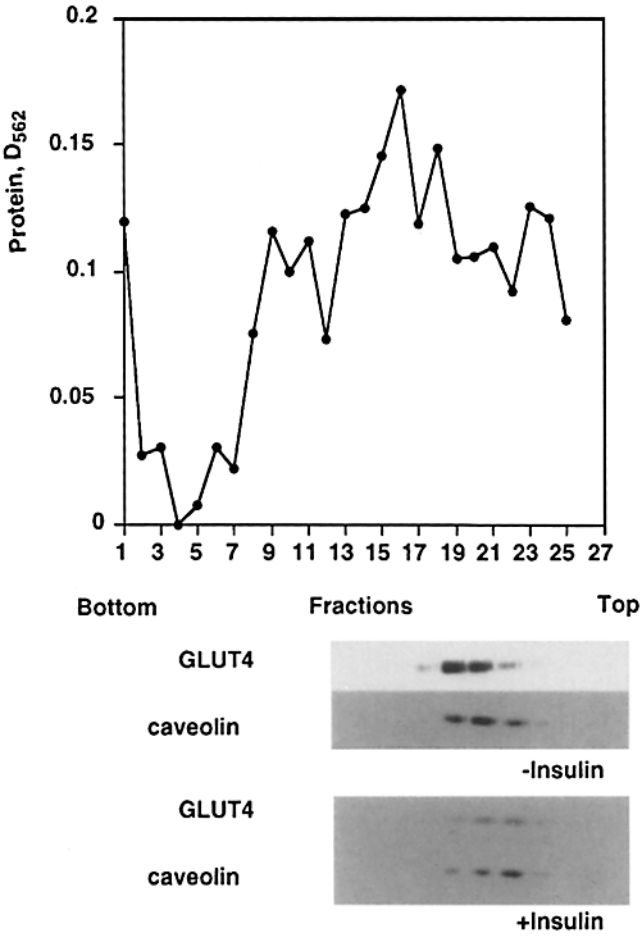

Figure 5. Caveolin- and GLUT4-containing vesicles have similar densities in sucrose gradients. LM (0.11 mg) from insulin-treated and -untreated adipocytes were fractionated in a 10–50% sucrose gradient as described in Materials and Methods. After centrifugation, odd fractions were analyzed by Western blotting as in Figs. 2 and 3.

eolin-containing vesicles and GLUT4-containing vesicles is considerably decreased after insulin administration to cells. It has been shown in numerous studies, that for the latter protein, insulin causes translocation of vesicles to the cell surface and their fusion with the plasma membrane. This process can be reliably quantitated and amounts to a 10–20-fold increase in cell surface GLUT4 (for review see Birnbaum, 1992; James and Piper, 1994; Mueckler, 1994; Stephens and Pilch, 1995). In contrast, the amount of caveolin in the plasma membrane of basal adipocytes is very high and the relatively small increase in plasma membrane caveolin caused by fusion with caveolin-containing intracellular vesicles amounts to a 15–20% increase. Nevertheless, the qualititatively similar translocation behavior of caveolin and GLUT4 seen in the previous three figures suggested that they might be colocalized in the same intracellular vesicles.

Thus, we immunoadsorbed GLUT4-containing vesicles from the LM fraction with 1F8 monoclonal antibody immobilized on polyacrylamide beads. As a control, we used nonspecific mouse IgG immobilized on the same beads at the same protein concentration, and neither protein binds to these beads. Fig. 6 represents the results of two independent experiments, which differ in the ratio between vesicles and immunobeads. Under conditions where >90% of the GLUT4-containing vesicles are adsorbed (bound to 1F8), no detectable caveolin is colocalized in the GLUT4 vesicles, rather 75–80% of this protein (and only 8% of GLUT4) is found in vesicles not bound to antibody (Fig. 6 *A*). Even if the ratio between the original LM and immunobeads is decreased five times (13 μg instead of 53 μg of LM for 10 μl of 1F8/IgG-beads), we still see ~5% of GLUT4 (and the same 75% of caveolin) in the supernatant (Fig. 6 *B*). We conclude that ~5% of GLUT4 in our preparation is inaccessible for interaction with 1F8-beads; these GLUT4 molecules are probably incorporated in some other structures, different from the "classical" vesicles.

Although practically no caveolin is bound to 1F8-beads in these immunoadsorption experiments, we usually see less caveolin in the supernatant than in the original material. This is most probably explained by the fact that some caveolin-containing vesicles are nonspecifically trapped by the beads, and then removed by the subsequent five washing steps. However, if the autoradiogram is greatly overexposed, a small amount (ca. 2% or less) of caveolin can be found associated with the GLUT4 vesicles (data not shown). Given that the vast majority of intracellular caveolin is excluded

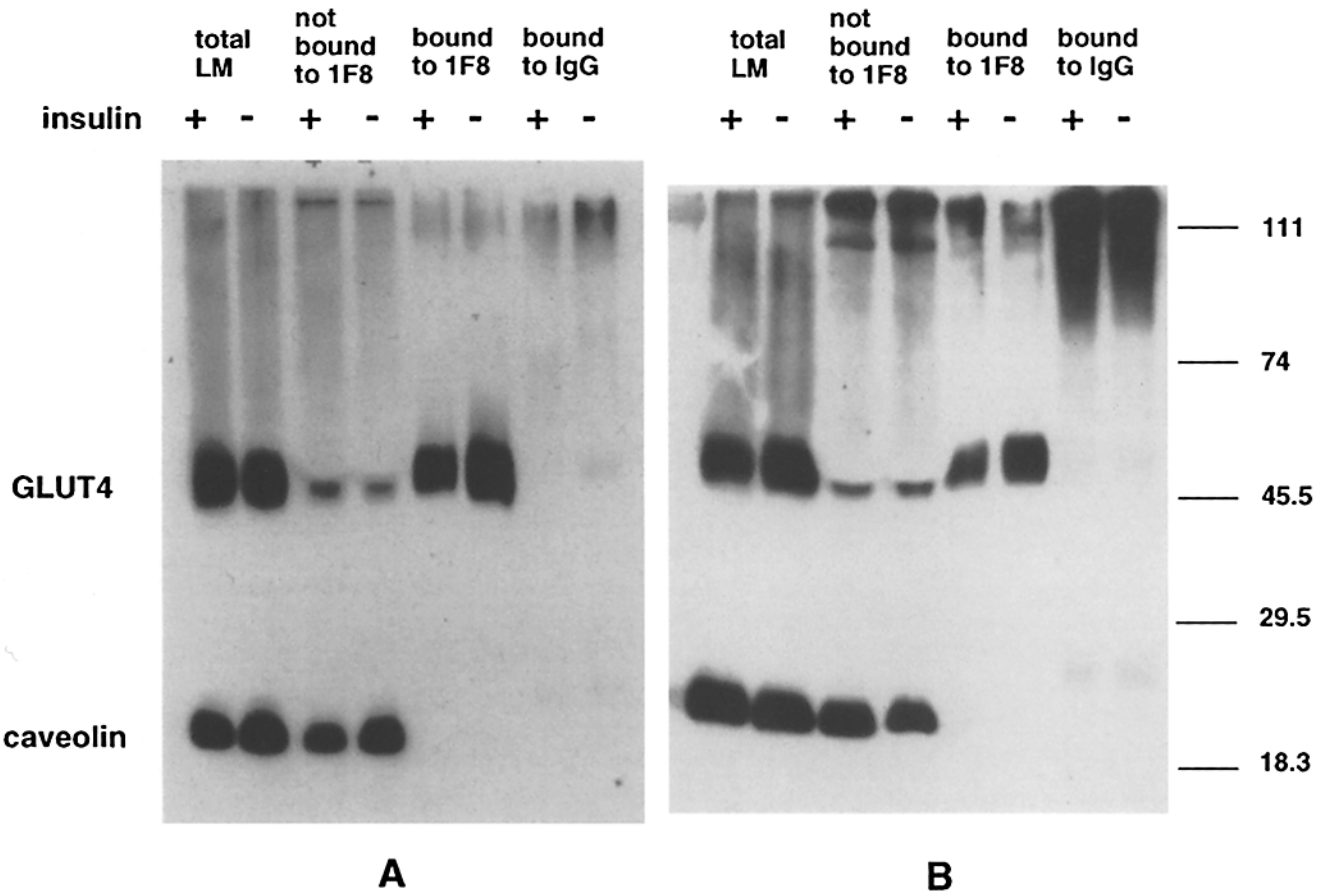

Figure 6. Caveolin is not present in GLUT4-containing vesicles. LM membrane (0.53 mg protein for *A*, 0.165 mg protein for *B*, both in 0.25 ml of PBS with protease inhibitors) from insulin-treated and -untreated adipocytes was immunoadsorbed with 0.1 ml (*A*) and 0.125 ml (*B*) of settled immunobeads as described in Materials and Methods. Elution of protein from the beads was performed with 0.4 ml of Laemmli sample buffer without 2-mercaptoethanol. Total LM (30 μg of each), supernatants of immunoadsorption (30 μg of each), and eluates (30 μl for A and 90 μl for B) were analyzed by electrophoresis in a 10% gel and Western blotting with anti-caveolin antibodies and 1F8. Molecular masses of the prestained protein standards (Gibco) are shown on the right. These experiments are representative of six such experiments which gave essentially identical results.

from GLUT4-containing vesicles, it seems highly unlikely that caveolin is involved in the formation and function of these structures.

It is known that caveolin can form detergent-insoluble complexes with other proteins, a phenomenon which may potentially be important for the structural organization of cellular exo- and endocytic machinery (Lisanti et al., 1993; Sargiacomo et al., 1993; Kurzchalia et al., 1992). Therefore, as another way to check for association of caveolin with GLUT4, we treated light microsomes with Triton X-100 and analyzed the solubilized protein in two ways. First, immunoprecipitation of either GLUT4 or caveolin with their respective antibodies did not reveal any coimmunoprecipitation (Fig. 7). Secondly, after fractionation of Triton-solubilized LM in sucrose gradient, caveolin is found in 8S aggregates (Fig. 8) roughly corresponding to a molecular mass of 160 kD, which is in excellent agreement with the original observation by Glenney (1989). We saw no overlap between the sedimentation patterns of detergent-solubilized caveolin and GLUT4, or other known proteins of GLUT4-containing vesicles such as GTV3/SCAMPs (Thoidis et al., 1993; Laurie et al., 1993) and small GTP-binding proteins (Cormont et al., 1991). When solubilized in detergent, these vesicular components sediment as monomers in the zone corresponding to their predicted molecular mass (data not shown). Thus, by several independent criteria, we see no evidence that intracellular caveolin and GLUT4 interact in any way.

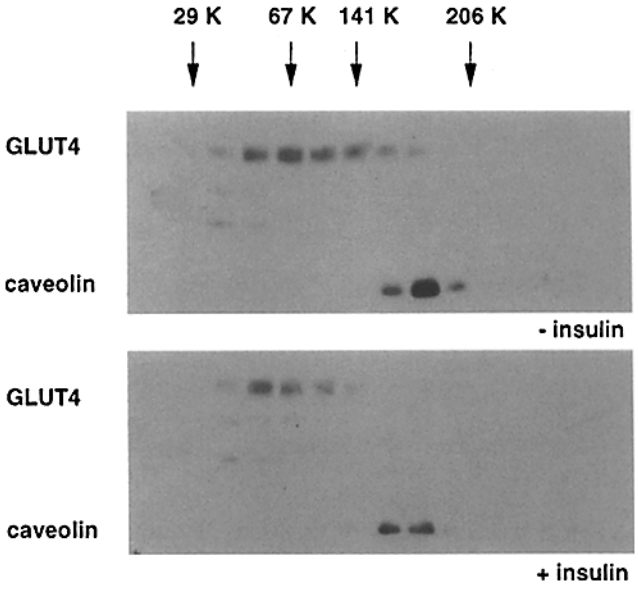

Figure 8. Sedimentational analysis of Triton-solubilized GLUT4 and caveolin. Triton-solubilized LM (0.11 mg) from insulin-treated and -untreated adipocytes was fractionated in a 5–20% sucrose gradient, as described in Materials and Methods. Carbonic anhydrase (29 kD), BSA (67 kD), yeast alcohol dehydrogenase (141 kD), and β-amylase (206 kD) were centrifuged in parallel gradients as standards. After centrifugation, odd fractions were analyzed by electrophoresis and Western blotting with anti-caveolin antibodies and 1F8.

Discussion

The possible physiological role of caveolae in the endocytosis and transport of small molecules, and in the formation of signal transduction complexes, has been the subject of much recent experimental activity as reviewed by van Deurs et al. (1993), Anderson (1993), and Kurzchalia et al. (1994). However, despite these reports, it is not at all clear what major physiological function(s) is performed by caveolae. Much evidence in this regard derives from the observation that Triton X-100 insoluble complexes contain the protein caveolin, the putative structural basis for caveolae, along with receptors and other signal transduction proteins (Sargiacomo et al., 1993; Lisanti et al., 1993; Shenoy-Scaria et al., 1994; Chun et al., 1994). However, similar Triton insoluble complexes can be isolated that are devoid of caveolin (Fra et al., 1994), and the percentage of total signal transducer molecules found in the presumed caveolae has not been determined (Sargiacomo et al., 1993; Chun et al., 1994). The observation that GPI-anchored cell surface proteins are clustered in caveolae, as a consequence of their specific apical surface targeting in epithelial cells, has been described as a likely artifact of antibody clustering (Mayor et al., 1994). Regardless of these controversies, it is clear from morphological data (Fan et al., 1983) as well as caveolin expression data (Fig. 1 and Scherer et al., 1994) that caveolae are particularly abundant in tissues that regulate organismal glucose homeostasis, namely muscle and fat. The fact that the expression of caveolin, and GLUT4 as well, are induced during differentiation of cultured adipocytes (Fig. 2 and Scherer et al., 1994) together with the coordinate downregulation of these two proteins by TNF (Stephens, J. M., K. V. Kandror, and P. F. Pilch, unpublished data), suggests that caveolin may be essential for the formation of the mature adipocyte phenotype, particularly, its insulin-regulated glucose transport.

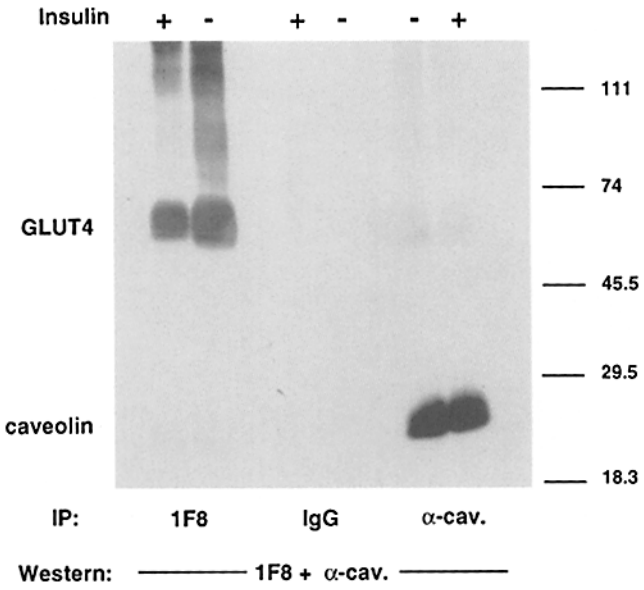

Figure 7. Immunoprecipitation of GLUT4 and caveolin from Triton-solubilized LM. Triton-solubilized LM (0.7 mg) from insulin-treated and -untreated adipocytes were immunoprecipitated with 1F8-beads, polyclonal anti-caveolin antibodies, and nonspecific rabbit IgG cross-linked to protein A-Trisacryl. Eluates were analyzed by electrophoresis in a 10% gel and Western blotting with anti-caveolin antibodies and 1F8. Molecular masses of the prestained protein standards (Gibco) are shown on the right.

However, our immunoadsorption data and sedimentational analysis (Figs. 6, 7, and 8) show there is essentially no caveolin in the GLUT4-rich vesicles either before or after cellular exposure to insulin. Intracellular caveolin does not form large detergent insoluble complexes with GLUT4 or other translocatable proteins. Hence, we can rule out a direct role for caveolin in the trafficking and formation of this particular population of transport vesicles. While this paper was in preparation, Scherer et al. (1994) published studies similar to ours on the expression and role of caveolin in cultured adipose cells as well as in acutely isolated rat adipocytes. They too observed the induction of caveolin synthesis during differentiation of 3T3-L1 cells, and our data confirm these results. However, we disagree strongly with their contention that caveolin and GLUT4 are colocalized to any substantial degree in the same type of vesicles. This issue is of crucial importance for evaluating the possible role of caveolin for structural organization of glucose transport in fat cells. Scherer et al. (1994) and ourselves have demonstrated the partial cosedimentation of caveolin- and GLUT4-containing material in sucrose gradients. However, we have also seen essentially identical sedimentation behavior for GLUT1-containing vesicles of fat cells (Kandror et al., 1995) and this protein is known to be excluded from GLUT4 vesicles by immunoadsorption experiments (Zorzano et al., 1989; Kandror et al., 1995). This latter type of experiment is crucial, and although Scherer et al. (1994) have visualized a caveolin band in immunoadsorbed GLUT4-containing vesicles, they present no data on the efficiency of their immunoadsorption procedure. Hence, they cannot make any quantitative conclusions on the degree of colocalization of the two proteins. In fact, we too can visualize some caveolin adsorbed with GLUT4 vesicles using the same, extremely sensitive ECL system used by Scherer et al. (1994) but only under conditions where all other signals are grossly overexposed (data not shown). We believe, therefore, that no meaningful amount of caveolin is present in GLUT4-containing vesicles. Immunoprecipitation of either GLUT4, or caveolin from Triton-solubilized LM gives evidence that GLUT4 does not form detergent-stable complexes with caveolin (Fig. 7).

It has been previously shown that GLUT4 is confined to the planar parts of rat adipocyte plasma membrane and is either excluded from caveolae (Voldstedlund et al., 1993) or is minimally present there (Slot et al., 1991*a*,*b*). Also, it is known that there is very little caveolin in the relatively featureless plasma membrane outside of caveolar invaginations (Rothberg et al., 1992; Dupree et al., 1993), the precise area where GLUT4 is found after insulin stimulation (Voldstedlund et al., 1993). All these data are complementary to our present results and, taken together, strongly suggest that the caveolin-containing membrane systems in adipocytes are structurally separated from the insulin-sensitive glucose-transporting machinery.

There is considerable evidence that GLUT4 containing vesicles may represent an example of a unique storage vesicle/endosome distinct from a classical endosome such as that involved in the endocytosis of insulin (Herman et al., 1994; Kandror et al., 1995). In support of this postulate, membrane fractionation of insulin-treated fat cells reveals that glucose transporters and insulin receptors are found in distinct fractions with minimal overlap (Kelly and Ruderman, 1993). With regard to GLUT4 vesicles, they also exclude GLUT1 (Zorzano et al., 1989; Kandror et al., 1995), the *trans*-Golgi marker, TGN38 (Martin et al., 1994) and ca. 90% of the IGF2 receptor (Zorzano et al., 1989; Kandror, K. V., and P. F. Pilch, unpublished). When vesicles are isolated from muscle and fat are compared, after very different tissue homogenization conditions, they are identical in apparent composition, in their sedimentation constants, buoyant densities, and their shift to higher values these last two parameters in response to insulin (Kandror et al., 1995). These vesicles possess two unique markers, GLUT4 and a tissue-specific aminopeptidase , gp160 (Kandror and Pilch, 1994*a*,*b*; Kandror et al., 1994) that cycle to and from the plasma membrane in a coordinate fashion, and are only found in the 3% of the total LM protein that represents the GLUT4-enriched vesicles. Finally, the kinetics of cycling, the presence of aminopeptidase activity and the abundance of a specific transport protein, namely the water channel, aquaporin CD, are features of antidiuretic hormone-sensitive vesicles in the kidney collecting duct (Harris et al., 1994) which are very similar to those described for insulin-sensitive GLUT4 vesicles. These water channel containing vesicles have also been described as a unique type of storage vesicle/endosome (Bradbury and Bridges, 1994).

What then is the possible role of caveolin in adipocytes and other insulin sensitive tissues that activate glucose transport and why are GLUT4 and caveolin expression regulated in a coordinate fashion? We hypothesize that because caveolin is structurally excluded from the pathways of GLUT4 trafficking, it may directly participate in this process by regulating access to the adipocyte surface, thus defining areas inaccessible for fusion with GLUT4-containing vesicles. This would facilitate the coordinate cycling of GLUT4 vesicle proteins, and alleviate the need for multiple sorting steps after endocytosis. However, the mechanism by which this might be accomplished is not at all clear. We are currently further analyzing GLUT4 vesicle composition, and we are searching for additional proteins that are expressed in a tissue-specific manner similar to GLUT4, gp160, and caveolin in order to address this issue.

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