Caveolin-2 Is Targeted to Lipid Droplets, a New "Membrane Domain" in the Cell

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Abstract. Caveolin-1 and -2 constitute a framework of caveolae in nonmuscle cells. In the present study, we showed that caveolin-2, especially its β isoform, is targeted to the surface of lipid droplets (LD) by immunofluorescence and immunoelectron microscopy, and by subcellular fractionation. Brefeldin A treatment induced further accumulation of caveolin-2 along with caveolin-1 in LD. Analysis of mouse caveolin-2 deletion mutants revealed that the central hydrophobic domain (residues 87–119) and the NH$_2$-terminal (residues 70–86) and COOH-terminal (residues 120–150) hydrophilic domains are all necessary for the localization in LD. The NH$_2$- and COOH-terminal domains appeared to be related to membrane binding and exit from ER, respectively, implying that caveolin-2 is synthesized and transported to LD as a membrane protein. In conjunction with recent findings that LD contain unesterified cholesterol and raft proteins, the result implies that the LD surface may function as a membrane domain. It also suggests that LD is related to trafficking of lipid molecules mediated by caveolins.

Key words: lipid droplet • caveolin-2 • caveolin-1 • membrane domain • brefeldin A

Introduction

Caveolae are small invaginations of the plasma membrane, and are assumed to play important roles in signal transduction, cholesterol transport, and endocytosis (Anderson, 1998; Fujimoto et al., 1998; Okamoto et al., 1998). Caveolins are essential for the above functions and form the structural framework of caveolae (Parton, 1996). Among caveolin family members, caveolin-1 is sufficient to induce caveolae formation, binds to cholesterol, and interacts with signaling molecules. Caveolin-3, expressed in limited kinds of cells, is thought to play a similar role as caveolin-1. In contrast, the function of caveolin-2 has not been defined in detail, except that recent results implied its involvement in forming deep caveolae invagination (Scheiffele et al., 1998; Fujimoto et al., 2000).

In the present study, we found that caveolin-2 can be targeted to the surface of lipid droplets (LD).[1] LD are found not only in adipose cells, but also in many other cells under various conditions. A notable example is the foam cell in the atheromatous plaque, but the functional significance of LD has not been known. Our result is consistent with the assumption that the LD surface is a "half membrane" consisting of a phospholipid monolayer. Interestingly, LD were reported to contain unesterified cholesterol (Prattes et al., 2000) and raft proteins related to signaling (Yu et al., 1998, 2000). Based on the findings, we propose that the surface of LD functions as a membrane domain.

Materials and Methods

Cell Culture

Cells were grown in DME with 10% FCS [HepG2, normal rat kidney (NRK), and human fibroblast], in Ham's F-12/DME with 10% horse serum and 2.5% FCS (Y-1), or in Ham's F-12/Coon's medium with 5% horse serum and hormones (FRTL-5). To induce LD formation, cells were cultured with oleic acid complexed with BSA (OA/BSA) (Brasaemle et al., 1997) for 2 d.

Transfection

Transient cell transfections were done by calcium phosphate or DEAE-dextran methods. Stably transfected cells were made using LipofectAMINE 2000 reagent and selected by G418 (GIBCO BRL). Caveolins were cloned by reverse transcriptase PCR from human fibroblasts and 3T3-L1 cells; the standard PCR was used to construct various mutant proteins, which were controlled by sequencing. Enhanced green fluorescent protein (EGFP) was tagged to the NH$_2$ terminus of caveolin-2 molecules by using pEGFP vectors (CLONTECH Laboratories, Inc.).

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[1]*Abbreviations used in this paper:* BFA, brefeldin A; EGFP, enhanced green fluorescent protein; LD, lipid droplets; NRK, normal rat kidney; OA, oleic acid.

Antibodies

Antibodies to caveolin-2 (Transduction Laboratories), caveolin-1 (Santa Cruz Biotechnology, Inc.), adipophilin (Progen), and Na$^+$/K$^+$-ATPase (Affinity Bioreagents) were purchased. Antibodies to GM130 (Nakamura et al., 1995), ER (Louvard et al., 1982), and calreticulin (Ioshii et al., 1995) were donated by Dr. Nobuhiro Nakamura (Kanazawa University, Kanazawa, Japan), Dr. Daniel Louvard (Institut Curie, Paris, France), and Dr. Toshimichi Yoshida (Mie University, Mie, Japan), respectively. Secondary antibodies were bought from Jackson ImmunoResearch Laboratories and Amersham Pharmacia Biotech.

Subcellular Fractionation and Western Blotting

LD were isolated as described from the nitrogen cavitate (Yu et al., 2000). Fractions precipitated with TCA were separated by SDS-PAGE, blot to nitrocellulose paper, and probed by antibodies. After incubation with HRP-conjugated antibodies, the reaction was visualized by chemiluminescence. The protein content was measured by BCA assay (Pierce Chemical Co.).

Immunofluorescence Microscopy

To localize caveolin-2, cells fixed with 3% formaldehyde were permeabilized either with Triton X-100 for labeling LD and the Golgi, or with methanol for labeling the plasma membrane. LD was stained with Sudan III (Chroma) and observed as orange-red fluorescence. Some cells were treated with BFA (Calbiochem) and nocodazole (ICN Biomedicals) before fixation. Samples were observed by conventional or by confocal fluorescence microscopy.

Immunoelectron Microscopy

Cells were fixed in 1% formaldehyde for 15–30 min. For ultrathin cryosections, cell pellet infiltrated with 2.3 M sucrose was processed by the Tokuyasu method (Tokuyasu, 1986). For freeze-fracture replicas, cell pellet infiltrated with 30% glycerol was freeze-fractured, and platinum/carbon replicas were labeled as described previously (Fujimoto, 1995).

Results

Caveolin-2 Is Targeted to LD

HepG2 lacks caveolins and has prominent LD in a standard culture condition. Human caveolin-2β transfected to HepG2 showed round-shaped labeling, which was revealed to be around LD by Sudan III staining (Fig. 1 a). Labeling for caveolin-2 was seen discontinuously around LD. Four cell lines stably expressing caveolin-2β gave equivalent results, and clone A-8 was used for most experiments. Expression of caveolin-2 did not appear to change the size and number of LD in HepG2. In contrast, human caveolin-1 transfected to HepG2 was seen in the cell surface and not related to LD (Fig. 1 b). Transfected caveolin-2 also occurred in LD of other cell types. In FRTL-5, LD were absent in a normal culture condition, and transfected caveolin-2 was seen in the Golgi as reported (not shown; Mora et al., 1999). But when LD were induced by OA/BSA, caveolin-2 was observed around them (Fig. 1 c).

Caveolin-2β in HepG2 was localized almost exclusively in LD, and the labeling coexisting with that of GM130, a Golgi protein (Nakamura et al., 1995) was scarce (Fig. 1 d). On the other hand, transfection of full-length caveolin-2 cDNA gave rise to prominent Golgi labeling, and LD were labeled only occasionally (Fig. 1 e). Western blotting showed predominant caveolin-2α and little caveolin-2β in those cells (not shown). In any cell line described above, neither anti–GM130 nor anti–ER antibody labeled LD (not shown).

Localization of caveolin-2 in LD was confirmed by immunoelectron microscopy. In cryosections, the labeling
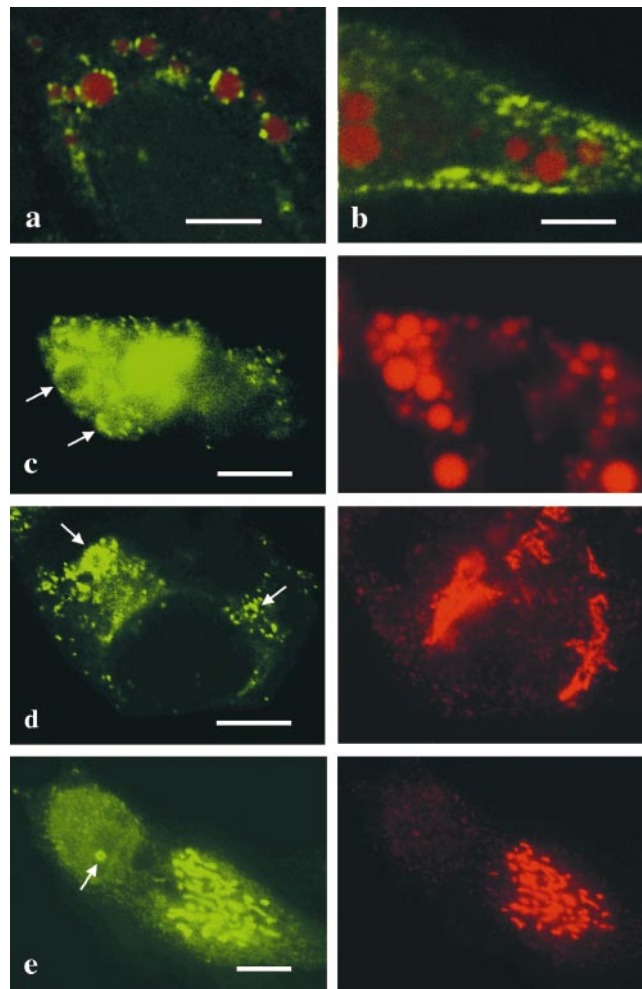

Figure 1. Confocal microscopy of HepG2 stably expressing caveolin-2β (a) and caveolin-1 (b). Caveolins (green), LD stained by Sudan III (red). Caveolin-2 is seen around LD discontinuously, whereas caveolin-1 is along the cell edge, and not related to LD. (c) FRTL-5 transiently transfected with full-length caveolin-2 cDNA and cultured with OA/BSA for 2 d. Caveolin-2 (green) is observed around LD (red). HepG2 stably transfected with caveolin-2β (d) or full-length caveolin-2 (e) cDNA was labeled for caveolin-2 (green) and GM130 (red). The labeling of caveolin-2β overlapping with that of GM130 is scarce (d), whereas caveolin-2α, the predominant isoform in the cell, is mostly localized in the Golgi (e). Arrows indicate labeling around LD. Bars, 10 μm.

was localized along the LD rim, and not in adjacent organelles (Fig. 2 a). Notably, most gold particles were seen in small clusters. The localization in LD was even more evident in freeze-fracture replicas. LD was identified by the onion-like morphology; the labeling for caveolin-2 also occurred in clusters and was seen only on the P, but not the E, face of LD (Fig. 2 b).

The LD localization was also confirmed by subcellular fractionation (Fig. 2 c). The top two fractions obtained by ultracentrifugation contained only 1.3% of the total protein, and were devoid of markers for ER, Golgi, and plasma membrane. But the fractions were positive for adipophilin, an LD-specific protein, and contained a significant amount of caveolin-2.
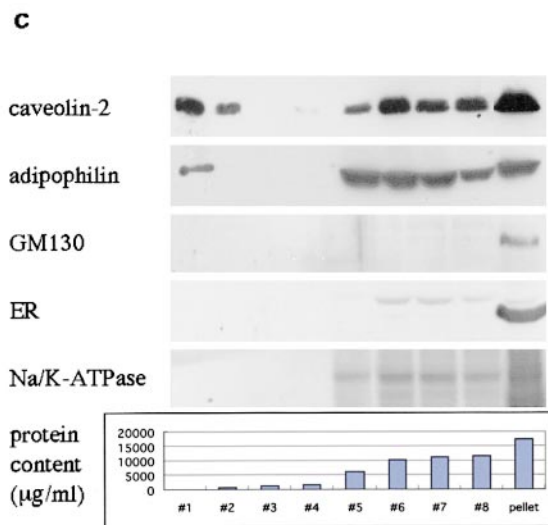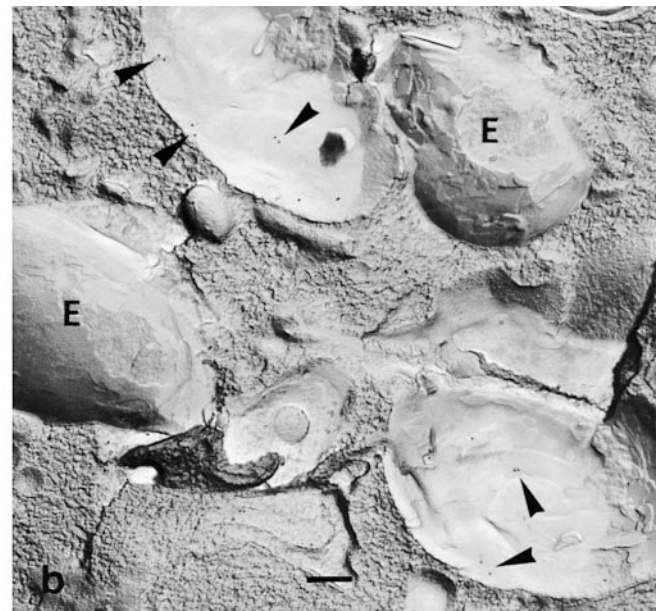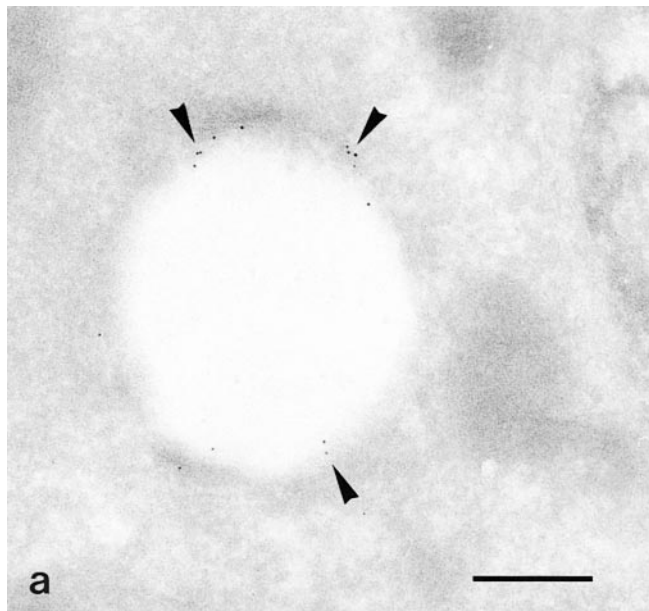
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Figure 2. (a and b) Immunoelectron microscopy of HepG2 expressing caveolin-2β (clone A-8). Bar, 500 nm. (a) Ultrathin cryosection. Gold particles labeling caveolin-2 are observed in the rim of LD in small clusters (arrowheads); the content of LD appears vacant. (b) Freeze-fracture replica. Gold particles for caveolin-2 are observed in clusters (arrowheads) on the P face of LD, which show an onion-like morphology. The E face of LD (E) is devoid of labeling. (c) Western blotting of subcellular fractions obtained from HepG2 clone A-8. The graph shows the protein content of the fractions. The top two fractions (#1 and #2) contained little protein, but showed positive signals for caveolin-2 and adipophilin. GM130, 66-kD protein, and Na$^+$/K$^+$-ATPase (Golgi, ER, and plasma membrane markers, respectively) are found only in the bottom fractions.

Caveolin-2 was seen in LD even when caveolin-1 is coexpressed. EGFP-mouse caveolin-2 transfected to NRK and Y-1 was recruited to LD (Fig. 3 a). The LD localization of caveolin-2 was also seen in HepG2 doubly transfected with untagged caveolin-1 and -2 (clone 6; Fig. 3, b and c). LD was found in most cells, but LD localization of caveolin-2 was not seen frequently. Notably, in cells showing caveolin-2 in LD, labeling of caveolin-1 was not observed in LD, but occurred in the plasma membrane. The result was confirmed by the lack of caveolin-1 signal in the LD fractions obtained from the doubly transfected HepG2 (Fig. 3 d).

Brefeldin A Causes Accumulation of Caveolins in LD

Neither immunofluorescence labeling nor Western blotting of subcellular fractions could detect endogenous caveolin-2 in LD of NRK, Y1, or human fibroblasts (not shown). Predominance of the α over the β isoform (Scherer et al., 1997) may be a reason for the negative result. However, when HepG2 expressing caveolin-2α was treated with 5 μg/ml brefeldin A (BFA), the LD distribution increased drastically within 2 h (Fig. 4 a). When BFA was applied to human fibroblasts loaded with OA/BSA, endogenous caveolin-1 and -2 were both labeled in LD (Fig. 4, b and c). The LD labeling became visible much earlier for caveolin-2 than for caveolin-1. The result showed that endogenous caveolin-2 can be detected in LD when normal trafficking is perturbed by BFA.

Interestingly, the BFA treatment also recruited caveolin-1 to LD in HepG2 expressing caveolin-1 alone (Fig. 4 d). But the redistribution of caveolin-1 to LD was slow and not detectable at 2 h. Even at 7 h, it was seen only in occasional cells, and most labeling remained on the cell surface.

Sequences Required for Targeting to LD

To identify molecular domains required for targeting to LD, mouse caveolin-2 mutants were constructed (Fig. 5 a) and transfected transiently to HepG2. First, to examine whether caveolin-2α goes to LD, the second methionine

Figure 3. (a) NRK, expressing EGFP–caveolin-2 along with endogenous caveolin-1 and -2, cultured with OA/BSA. EGFP-caveolin-2 (green) is seen around LD (red) (arrows). (b and c) HepG2 stably expressing caveolin-1 and -2 (clone 6). Caveolin-1 (red) and -2 (green) show colocalization in the plasma membrane (b), and only caveolin-2 (green) is observed around LD (red) in some cells (arrows) (c). Cells in b and c were treated with methanol and Triton X-100, respectively, to show the plasmalemmal (b) and intracellular (c) labeling. Bars, 10 μm. (d) Western blotting of subcellular fractions obtained from HepG2 clone 6. Only caveolin-2, but not caveolin-1, was detected in the LD fractions (#1 and #2).

Figure 4. (a) HepG2 stably transfected with full-length caveolin-2 cDNA was treated with BFA for 2 h. Caveolin-2 (green) became confined to LD (red) in most cells (arrows). (b and c) Human fibroblasts treated with BFA for 16 h. Not only caveolin-2 (b), but also caveolin-1 (c) (green) was observed around LD (red) (arrows). (d) HepG2 stably transfected with full-length caveolin-1 cDNA. After the BFA treatment for 7 h, caveolin-1 (green) was seen around LD (red) in occasional cells (arrows). Bars, 10 μm.

(residue 14) was replaced with leucine to abolish generation of the β isoform. The mutant (M14/L) showed the dual distribution in the Golgi and LD, proving that the α isoform can be distributed in LD (Fig. 5 b). Another mutant with glycine (residue 2) replaced with alanine (G2/A) was made to examine the possible influence of NH$_2$-terminal myristoylation on targeting. G2/A showed a distribution similar to the intact α isoform and thus the myristoylation does not account for the preferential Golgi localization (Fig. 5 b). The result suggests that the 13 amino acids specific to the α isoform may reduce the affinity to LD, or may be a strong signal for Golgi localization.

Second, NH$_2$ and COOH termini of caveolin-2 were truncated in various lengths. Because NH$_2$-terminal truncation mutants were not reactive with the anti–caveolin-2 antibody (Das et al., 1999), they were fused to the COOH terminus of EGFP (Fig. 5 c). Up to 69 residues from the NH$_2$ terminus could be deleted (70–162) without producing a notable difference from the intact caveolin-2β, whereas deleting the 70th tryptophan residue (71–162) abolished both LD and Golgi localization and produced diffuse cytosolic and nuclear distribution. On the other hand, COOH-terminal deletion caused a gradual change (Fig. 5 d). 12 residues could be truncated without a visible effect (1–150 and 14–150; not shown), but by deleting further residues (1–144 to 1–132 and 14–144 to 14–132), localization to LD and Golgi became indistinct, and the cytoplasmic labeling in the network pattern increased. The cytoplasmic labeling showed colocalization with calreticulin, an ER lumenal protein, implying

that it is derived from ER; moreover, by treating cells with 10 μM nocodazole for 2 h, labeling for the caveolin-2 mutants and calreticulin showed a similar retraction from the cell edge (Fig. 5 d; Kogo et al., 1997). When 35 residues from the COOH terminus were deleted (1–127 and 14–127), both LD and Golgi labeling were almost invisible.

Third, when the central hydrophobic domain (87–119) was deleted from either α or β isoform, the mutants were found in the Golgi, but not in LD (Fig. 5 e). Even when cells were treated with BFA for 2 h, the labeling in LD was hardly observed (not shown). Fourth, EGFP tagged to the NH$_2$ terminus alone (1–86), the COOH terminus alone (127–162), or the hydrophobic domain alone (87–122) showed cytosolic distribution (not shown). The result indicates that the hydrophobic domain is important, but not sufficient, for the LD localization.

Discussion

LD as a Membrane Domain

The present study showed that caveolin-2, especially its β isoform, could localize in LD. Caveolin-2 is different from the hitherto reported LD proteins (Londos et al., 1999; Syu and Saltiel, 1999) in that it exists as an integral membrane protein in the plasma membrane. Caveolin-2 as well as other caveolins are supposed to anchor to the membrane by the central hydrophobic domain with both NH$_2$ and COOH termini exposed to the cytoplasm (Parton, 1996). The result that the anti–caveolin-2 antibody, recognizing the NH$_2$-terminal segment (Das et al., 1999), decorated only the P face of LD in the freeze-fracture replica suggests that caveolin-2 takes the same orientation on the LD surface as in the membrane. It is consistent with the hypothesis that the LD surface is a half-membrane, or a phospholipid monolayer, in which amphiphilic lipid molecules exist with their hydrophilic and hydrophobic portions oriented toward the cytoplasm and the LD content, respectively (Zweytick et al., 2000).

The analysis of mutated caveolin-2 molecules supported the above assumption. First, the NH$_2$-terminal segment (residues 70–86) was required for membrane attachment, and its deletion abolished the localization in LD. Second, the COOH-terminal segment (residues 120–150) appeared necessary for the exit from ER, and its truncation caused an increased retention in ER and a decreased distribution in LD. These results indicate that both membrane attachment and exit from ER are necessary for distribution in LD. That is, caveolin-2 is likely to be translated in ER like caveolin-1 (Monier et al., 1995), and then transported to LD as a membrane protein. Two LD proteins, perilipin of mammalian adipocytes (Londos et al., 1999) and oleosin of plant seeds (Napier et al., 1996), are also synthesized in the membrane-bound polyribosome.

It is noteworthy that the labeling for caveolin-2 was not seen evenly in the LD surface, but was found in small clusters both in cryosections and in freeze-fracture replicas. We observed that the labeling for adipophilin shows the same pattern in the LD surface (Nakamura, N., and T. Fujimoto, unpublished observations). These results suggest that the whole LD surface may not be homogenous and that some segregation may occur in the half-membrane.
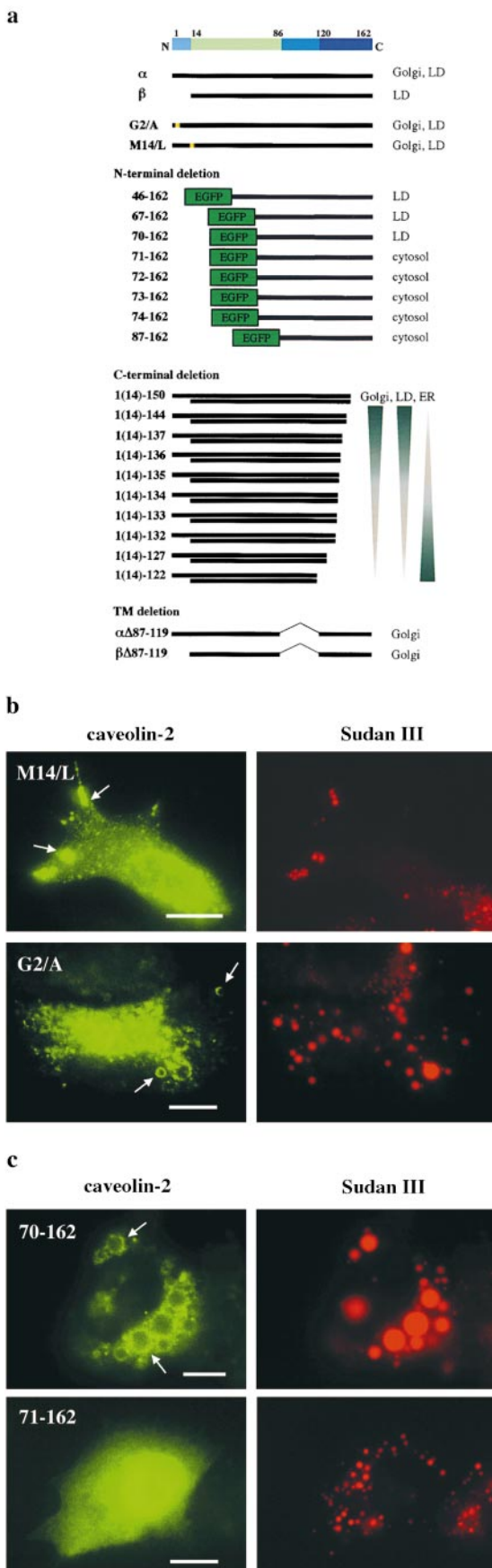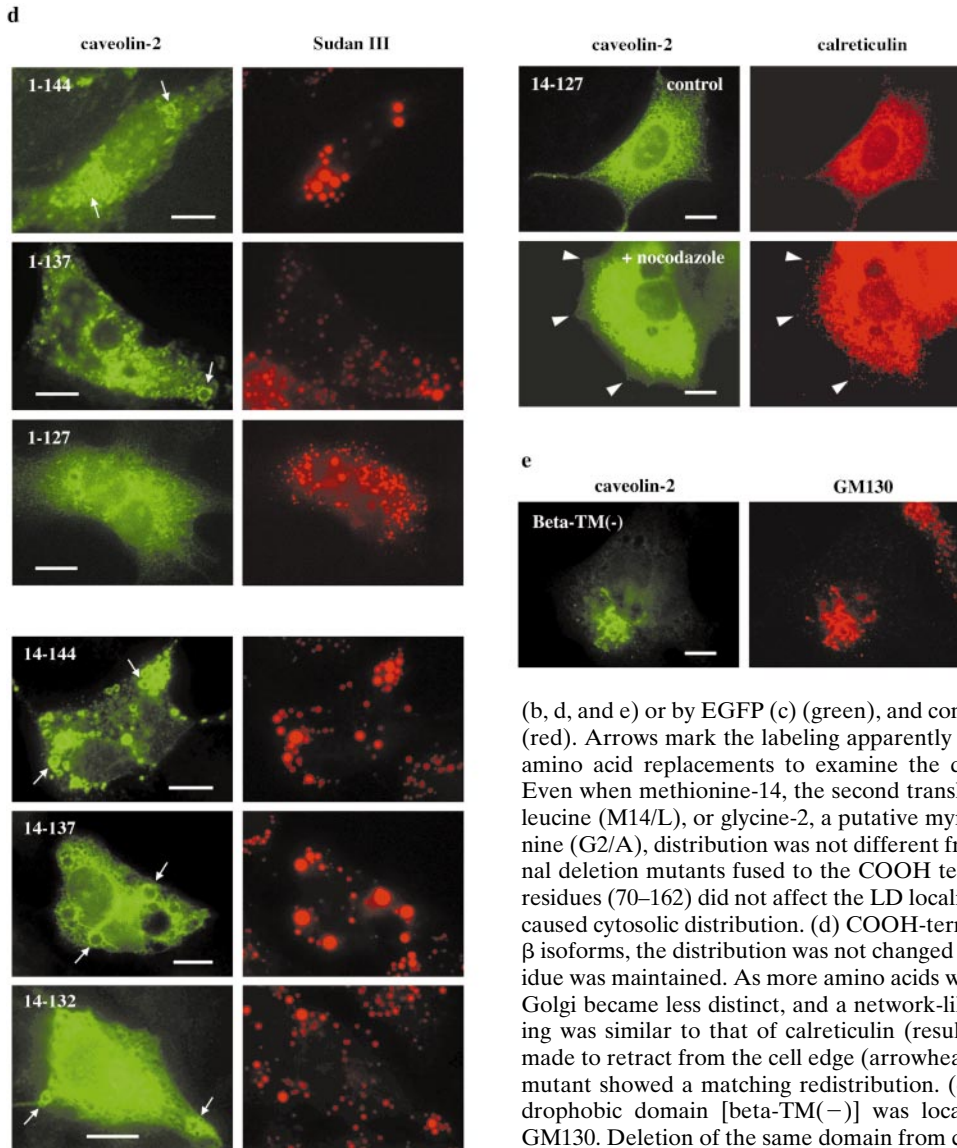

Figure 5 (continues on next page)

Figure 5. (a) A diagram of caveolin-2 mutants and their distribution on transient expression in HepG2. (b–e) Distribution of mutants was observed by anti–caveolin-2 labeling (b, d, and e) or by EGFP (c) (green), and compared with LD, Golgi, or ER markers (red). Arrows mark the labeling apparently encircling LD. Bars, 10 μm. (b) Single amino acid replacements to examine the difference between α and β isoforms. Even when methionine-14, the second translation initiation site, was replaced with leucine (M14/L), or glycine-2, a putative myristoylation site, was replaced with alanine (G2/A), distribution was not different from that of caveolin-2α. (c) NH₂-terminal deletion mutants fused to the COOH terminus of EGFP. Deletion of up to 69 residues (70–162) did not affect the LD localization, but further truncation (71–162) caused cytosolic distribution. (d) COOH-terminal deletion mutants. For both α and β isoforms, the distribution was not changed when the sequence up to the 150th residue was maintained. As more amino acids were deleted, localization in LD and the Golgi became less distinct, and a network-like labeling increased. The latter labeling was similar to that of calreticulin (result of 14–127 is shown). When ER was made to retract from the cell edge (arrowheads) by nocodazole, the labeling for the mutant showed a matching redistribution. (e) Caveolin-2β lacking the central hydrophobic domain [beta-TM(−)] was localized in the Golgi, labeled by anti–GM130. Deletion of the same domain from caveolin-2α gave the same result.

Interestingly, a recent study revealed the presence of unesterified cholesterol in LD (Prattes et al., 2000). Furthermore, LD of eosinophils contain raft proteins related to intracellular signaling, such as mitogen-activated protein kinase and Lyn (Yu et al., 1998, 2000). Our study extended the finding in that another raft molecule, caveolin-2, and possibly other caveolins, can be distributed in the LD surface. As a whole, these results suggest an intriguing possibility that LD might be a novel membrane domain where caveolins may functionally regulate signaling proteins.

Trafficking of Caveolins and LD

The mutagenesis study inferred that the hydrophobic domain of caveolin-2 (residues 87–119) is important for targeting to LD. Even when this domain was deleted, the protein bound to the membrane and exited from ER, but it was not seen in LD. Interestingly, oleosin and the hepatitis C virus core protein, which can associate with LD, have a hydrophobic domain flanked by hydrophilic/amphipathic domains, and its deletion eliminated LD localization (van Rooijen and Moloney, 1995; Hope and McLauchlan,

2000). Because sequence similarity does not exist between the two proteins and caveolin-2, it may be a stretch of hydrophobic amino acids that dictates localization in LD.

The recruitment of caveolin-1 in BFA-treated cells may also be due to the hydrophobic domain shared by all caveolins. However, caveolin-1 does not appear to have the same degree of affinity to LD as caveolin-2. First, in cells expressing caveolin-1 alone, its redistribution to LD after the BFA treatment occurred slowly and only in occasional cells. Second, without the BFA treatment, caveolin-1 was not found in LD even when overexpressed. The LD localization signal of caveolin-1 is likely to be less effective than that of caveolin-2.

Endogenous caveolin-2 was not detected in LD in the normal culture condition. It may be because caveolin-2α, expressed predominantly in most cells, is less efficient than caveolin-2β in LD targeting. But the accumulation of caveolin-2 in LD of BFA-treated cells indicates an alternative possibility; that is, caveolin-2, especially caveolin-2α, may stay in LD transiently, so that its distribution in LD can only be detected when the protein is expressed abundantly

or when its trafficking is perturbed. The morphological continuity of ER and LD (Blanchette-Mackie et al., 1995) and the persistence of LD labeling in BFA-treated cells indicates that caveolin-2 goes directly from ER to LD. But whether LD exists independently from the ER–Golgi pathway, or LD is an intermediate compartment between ER and the Golgi, is not known. The result of BFA experiments may simply indicate that an excessive amount of caveolin-2 in ER overflowed to LD. But BFA might also block other pathways (e.g., from LD to the Golgi) and have caused the LD retention. The two pathways are not mutually exclusive and need further studies for definition.

Trafficking of caveolins to LD is important because it may be linked to that of lipids. A testable question in this context is whether the lipid composition of LD changes by the presence of caveolins and/or by the BFA treatment, which is now being examined in our lab. It is also important to ask whether and how LD is related to the reported trafficking routes of caveolin-1 (Conrad et al., 1995; Uittenbogaard et al., 1998).

Concluding Remarks

Based on our results, we propose two new roles for LD. One is as a membrane domain possibly related to signaling and the other is as a transit point of lipid trafficking. LD have been thought to be a reservoir of lipids in a few specialized cells, but they occur in many cells in reality. Our result would be a starting point for further studies that should shed light on this well-known, but enigmatic round organelle.

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