

Caveolin-1 is transported to multi-vesicular bodies after albumin-induced endocytosis of caveolae in HepG2 cells

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Abstract

Caveolae-mediated endocytosis is a highly regulated endocytic pathway that exists in parallel to other forms of clathrin-dependent and -independent endocytosis. Internalized caveolae accumulate in intermediate organelles called caveosomes. Here we addressed the further fate of internalized caveolae by inducing caveolae-mediated uptake of albumin by HepG2 cells. We followed the route of internalized caveolin-1 by immunogold labelling of ultrathin frozen sections and by Western blot analyses of purified membrane fractions. Long-term (1 and 3 hrs) albumin treatment resulted in the appearance of albumin-containing caveolae in special multi-caveolar complexes (consisting of multiple caveolae clustered together) connected to the plasma membrane and caveosome-like structures in the cytoplasm. In addition, numerous CD63 (LIMP-1) positive late endosomes/multi-vesicular bodies were found positive for caveolin-1, suggesting that upon albumin incubation, caveolin-1 is endocytosed and enters the degradative pathway. Surprisingly, the number of caveolae at the plasma membrane increased after addition of albumin. This increase was blocked by cycloheximide treatment, indicating that albumin internalization also stimulates *de novo* protein synthesis, which is necessary for new caveolae formation. Together, our results show that during long-term albumin uptake, caveolin-1 travels to late endosomes and is replaced by newly synthesized caveolin-1 at the plasma membrane.

Keywords: caveolin-1 • albumin • multi-vesicular bodies

Introduction

Caveolae are detergent-resistant, highly hydrophobic membrane domains. The main protein components of caveolae are caveolins (caveolin-1, -2 and -3), which are essential for the formation and stability of caveolae [1]. The scaffolding domain of caveolin-1 can bind various signalling molecules [2] and thereby regulates their activity. Thus, caveolae are thought to function as pre-assembled signalling complexes or signalling organelles [3].

Although ultrastructural and biochemical studies have shown that caveolae are highly immobile structures [4], under special conditions they can pinch off from the plasma membrane [2]. This internalization is associated with receptor cross-linking and clustering in caveolae and reorganization of the actin cytoskeleton [2, 5].

After caveolar internalization, various ligands (SV40 virus, cholera toxin) accumulate in special cellular compartments, called caveosomes. Caveosomes were described as intermediate organelles of caveolae-mediated endocytosis. Until now, they are characterized only by the presence of caveolin-1 at their cholesterol- and sphingolipid-rich limiting membrane [6]. There are indications that caveolae can fuse with early endosomes in a Rab5-dependent manner [7, 8]. However, the question remains whether caveosomes or early endosomes are the only destination for internalized caveolin-1 [8] or whether caveolin-1 can

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travel to the late endosome–lysosome pathway, where it is degraded.

The albumin-binding adaptor protein (gp60) accumulates in caveolae and associates with caveolin-1 [9, 10]. When albumin binds to gp60, it triggers tyrosine phosphorylation of caveolin-1 and dynamin-2 *via* activation of c-Src kinase, resulting in the internalization of caveolae. These signal transduction steps occur shortly after the activation of gp60 [11].

In this study, we used albumin to provoke caveolae pinching off from the plasma membrane in the human hepatocellular carcinoma cell line HepG2 and investigated whether endocytotic caveolar carriers can interact with the classical endosomal degradative pathway. Since caveolar internalization is a slow process, we applied long-term (1 and 3 hrs) albumin incubation to induce internalization of caveolae. We followed the intracellular route of caveolin-1 on ultrathin cryosections and by purified membrane fractions and Western blotting.

Our data show that during long-time ligand internalization, caveolae intersect with the classical endocytotic pathway, where caveolin is degraded. *De novo* caveolin-1 synthesis is necessary to replace caveolae at the plasma membrane.

Materials and methods

Cells

The human hepatocellular carcinoma (HepG2) cell line was used in all experiments. Cells were grown on tissue culture flasks or 100-mm culture dishes in Dulbecco's modified Eagle's medium (Gibco, Invitrogen, Carlsbad, CA, USA) supplemented with 10% FBS (Gibco), 100 U/ml Penicillin 0.1 mg/ml Streptomycin (Sigma, Saint Louis, MI, USA).

Antibodies

Polyclonal anti-caveolin-1 (Transduction Laboratories, Exeter, UK), polyclonal anti-human albumin (Nordic Laboratories, Tilberg, the Netherlands), mouse-anti-CD63 antibodies (PeliCluster, Amsterdam, the Netherlands) and polyclonal anti-mouse IgG (obtained from Dako Laboratories, Carpenteria, CA, USA) were used for immunogold labelling on frozen sections. For electron microscopic localization of the primary antibodies on ultrathin cryo-sections, protein-A gold (d: 10 and/or 15 nm) was used (Cell Microscopy Center, Utrecht, the Netherlands). For Western blot analysis, polyclonal anti-caveolin-1 antibody (1:500) and monoclonal anti-syndecan-1 antibody (Dako Laboratories) (1:500) were applied.

Experimental procedures

Before the albumin internalization, confluent HepG2 cells were washed with phosphate buffer and then incubated for 24 hrs in serum-free media. After this albumin depletion, cells were treated with 30 mg/ml albumin (serum albumin–fraction V., 98% pure, Sigma Chemical Co.)

for 60 and 180 min. at 37°C. Protein synthesis was inhibited when 50 µg/ml cycloheximide (Sigma) was added to the 30 mg/ml albumin-containing culture medium, and the cells were incubated for 60 and 180 min. at 37°C.

Immunoelectron microscopy

Cells were fixed by adding 4% freshly prepared formaldehyde in 0.1 M phosphate buffer pH 7.4 to an equal volume of culture medium for 5 min., followed by post-fixation in 4% formaldehyde in 0.1 M phosphate buffer pH 7.4 without medium for 24 hrs at 4°C. Cells were stored until further processing in 1% formaldehyde at 4°C. Processing of cells for ultrathin cryosectioning and immunolabelling according to the protein A-gold method was done as described in [12]. In brief, fixed cells were washed with 0.05 M glycine in PBS, scraped gently from the dish in PBS containing 1% gelatin and pelleted in 12% gelatin in PBS. The cell pellet was solidified on ice and cut into small blocks. For cryoprotection, blocks were infiltrated overnight with 2.3 M sucrose at 4°C and afterwards mounted on aluminium pins and frozen in liquid nitrogen. To pick up ultrathin cryosections, a 1:1 mixture of 2.3 M sucrose and 1.8% methylcellulose was used [13].

Morphometry and statistical analysis

Twenty to twenty-two electron micrographs were randomly taken from each experimental group. The numbers of morphologically identified caveolae connected to the plasma membrane and under the plasma membrane (internalized vesicles) were counted. The surface/volume ratio was calculated according to Weibel *et al.* (1966) [14]. Results were expressed in number of vesicles per surface area of the cell. To quantitate the caveolin-1-containing multi-vesicular bodies/late endosomes, anti-CD63 and/or anti-caveolin-1 labelled organelles were counted in 10–15 randomly chosen individual cells. StatSoft Statistica 6.1 software (StatSoft Inc., Tulsa, OH, USA) was used for statistical analysis.

In case of each experimental group, the morphometrical measurements were done on sections immunolabelled on the same day.

Membrane purification and Western blot

For all experimental groups, confluent cultures of HepG2 cells grown in 100-mm culture dishes were used. Plasma membrane and intracellular membrane fractions were prepared according to Smart *et al.* (1995) [15] and Conrad *et al.* (1995) [16] at 4°C. Cells were washed and then pelleted in buffer A (0.25 M sucrose, 1 mM EDTA, 20 mM Tricine, pH: 7.8). Homogenate of cells was layered on top of 23 ml 30% Percoll in buffer A and centrifuged at 84,000g for 30 min. at 4°C in VAC602 ultracentrifuge. The low-density fraction containing plasma membrane, and the high-density fraction with Golgi complex, ER, lysosomes and endosomes were collected and analysed by immunoblotting with anti-caveolin-1. Protein concentrations were determined by Bio-Rad Bradford assay and same amount of protein (50 µg/ml) was layered on the top of 10% SDS gel. After blotting, anti-caveolin-1 or anti-syndecan-1 antibody was used, the secondary antibody (biotinylated goat anti-rabbit antibody or biotinylated horse anti-mouse antibody [Vector, Burlingame, CA, USA]) was diluted to 1:500. The immunoreactive proteins were detected by NovaRED Substrate Kit (Vector).

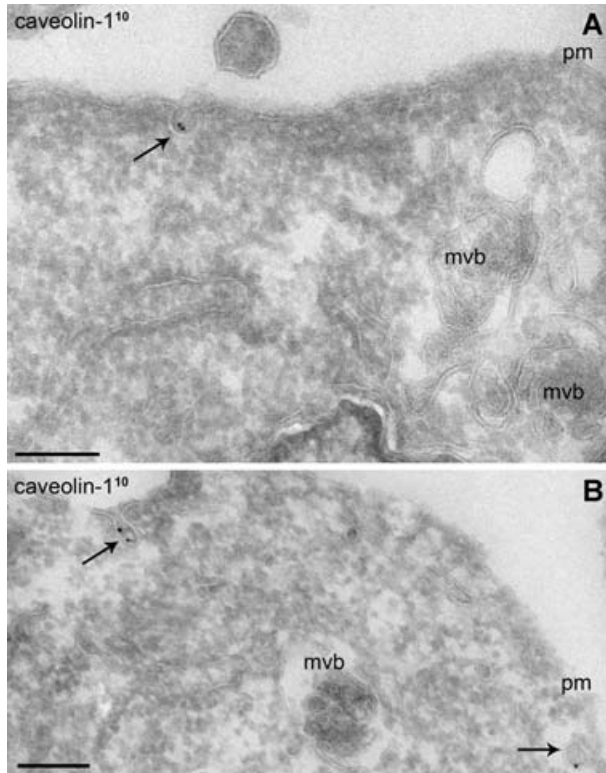


Fig. 1 Albumin-depleted HepG2 cells display low numbers of caveolae at the plasma membrane. Ultrathin cryosections of HepG2 cells immunogold labelled for caveolin-1 (10-nm gold). **A** and **B**: After 24-hr albumin depletion, only few caveolae are observed at the plasma membrane (pm) of HepG2 cells, which occur as separate entities. Late endosomes/multi-vesicular bodies (mvb) are devoid of caveolin-1. Bars, 200 nm.

Results

Albumin triggers formation and endocytosis of caveolae

Since caveolar internalization is a slow process, we applied long-term (1 and 3 hrs) albumin incubation to induce internalization of caveolae. We followed the intracellular route of caveolin-1 by immunogold labelling of ultrathin cryosections. In HepG2 cells grown under standard conditions (10% FBS containing medium), we consistently observed caveolae, displaying their characteristic flask-like appearance at the plasma membrane (data not shown). When cells were incubated in albumin-free medium for 24 hrs, the number of caveolae at the plasma membrane significantly decreased. They appeared as separate entities associated with the plasma membrane (Fig. 1).

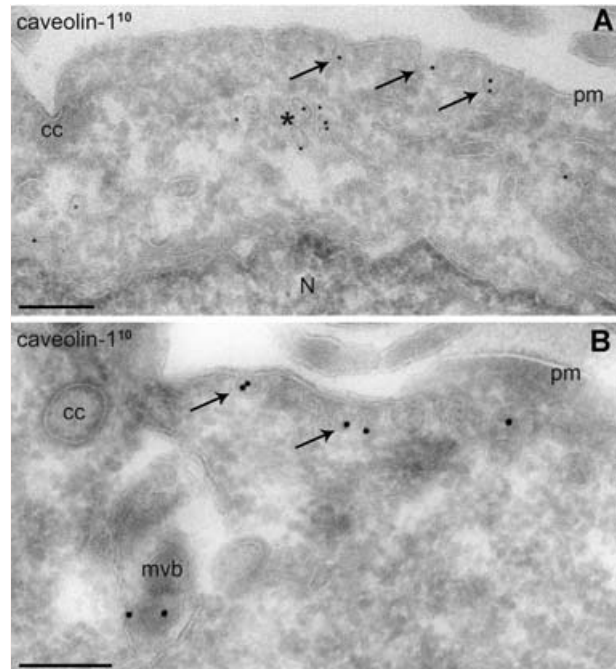


Fig. 2 Albumin incubation increases the number of caveolae at the plasma membrane. Ultrathin cryosections of HepG2 cells immunogold labelled for caveolin-1 (10-nm gold). **A** and **B**: One-hour incubation in albumin containing medium results in the appearance of numerous caveolae (arrows) along the plasma membrane (pm). The asterisk indicates a caveolar cluster in the cytoplasm. Note in **B**, the presence of caveolin-1 labelling in a multi-vesicular body (MVB). N = nucleus, cc = clathrin-coated pit and vesicle. Bars, 200 nm

After incubation in albumin-containing medium for 1–3 hrs, numerous caveolae appeared at both the plasma membrane and in the cytoplasm at a small distance from the plasma membrane (Fig. 2). This indicated that albumin induced the formation as well as internalization of caveolae. The increase in number of caveolae was already prominent when cells were exposed to albumin for 1 hr (Fig. 2). Quantitative analysis (Fig. 4) confirmed that albumin incubation induced a significant increase in the number of caveolae both at the plasma membrane and in the cytoplasm. The number and distribution of caveolae after 1- and 3-hrs albumin incubation remained approximately similar. Notably, albumin incubation did not change the number of coated vesicles at the plasma membrane.

In addition to the increase in number of caveolae, albumin incubation also induced the formation of multi-caveolar complexes consisting of multiple caveolae clustered together (Figs. 2 and 3). According to their morphology, and the presence of caveolin-1, these structures can be considered as caveosomes. Some caveolar clusters were seen in connection with the cell surface at the plane of the section (Fig. 3A). Others were found deeper in the cytoplasm, at a distance from the plasma membrane, suggesting

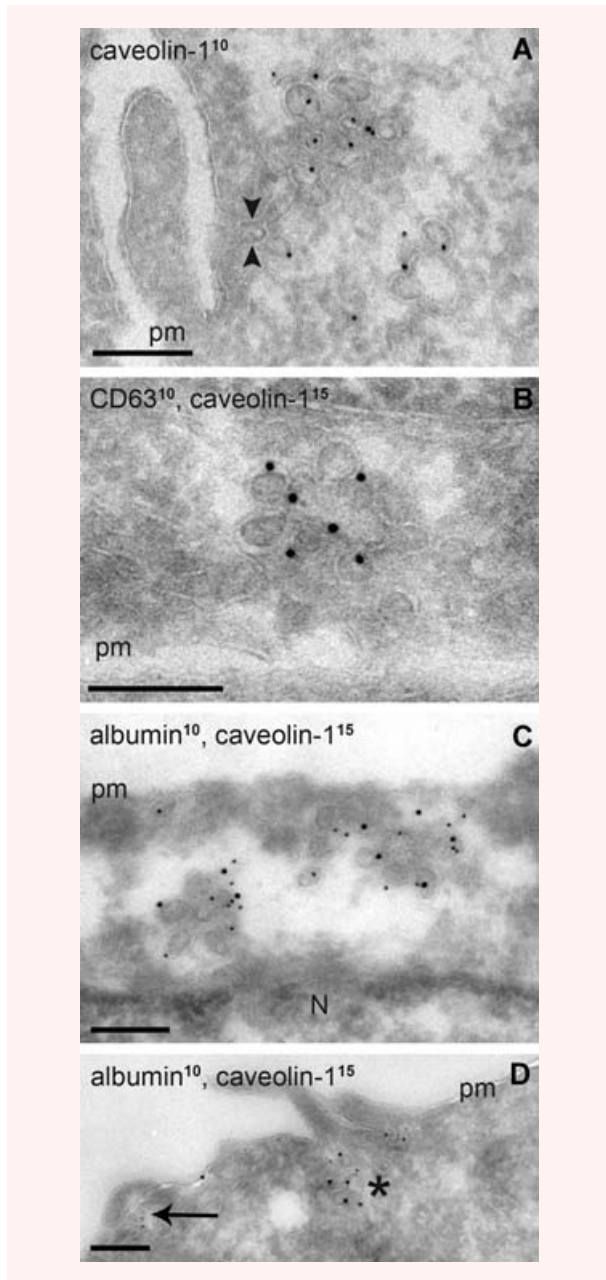


Fig. 3 The number of plasma membrane caveolae and internalized caveolae increase after albumin treatment. Morphometrical analysis. After 1-hr albumin incubation, the number of membrane-connected and pinched off caveolae significantly increases ($P < 0.005$ versus control [0-hr albumin]). After 3-hr incubation, there is only a small additional increase of caveolae. Albumin incubation had no influence on the number of clathrin-coated pits and vesicles along the plasma membrane.

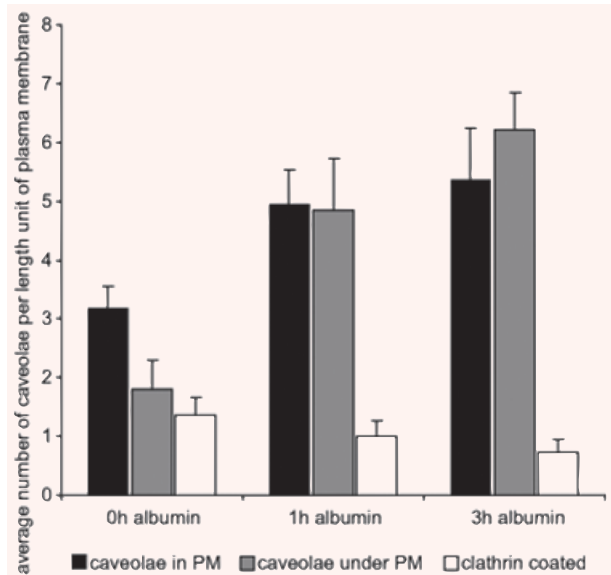


Fig. 4 Albumin incubation induces the appearance of caveolar clusters. During long-term internalization, multi-caveolar complexes (consisting of multiple caveolae clustered together) and caveosomes appear in the cytoplasm. **A:** Some of them are obviously connected to the plasma membrane (pm) via a narrow membrane-channel (arrowheads). **B:** CD63 is absent from these structures. **C:** Albumin can be readily localized in the caveolar clusters. **D:** Caveolin-1 and albumin colocalize in caveolar clusters (asterisk). N = nucleus. Bars, 200 nm.

that they are detached to form an independent structure (Fig. 3A, B and C). To gain more information on the role of these caveolae-based structures, we performed double-immunogold labelling for caveolin-1 and albumin, as well as for caveolin-1 and the late endosomal marker CD63. We found that albumin colocalized with caveolin-1 in single caveolae as well as in the multi-caveolar complexes (Fig. 3C and D), in agreement with their role in endocytosis. Notably, none of the caveolae-related structures contained the late endosomal marker protein CD63 (Fig. 3B). Together, these data show that albumin incubation induces the formation of more and more complex caveolar structures, which are actually involved in the uptake of albumin.

Albumin uptake drives caveolin-1 to the degradative pathway

Late endosomes or multi-vesicular bodies (MVB) can be identified by numerous intraluminal vesicles and the presence of CD63 (LIMP-1) antigen [17]. In HepG2 cells growing under control conditions (10% FBS containing medium), many CD63 positive MVBs are present in the cytoplasm. Only few of these MVBs also stained for caveolin-1 (Fig. 5A). After albumin incubation, the number of caveolin-1 containing CD63-labelled endosomes increased

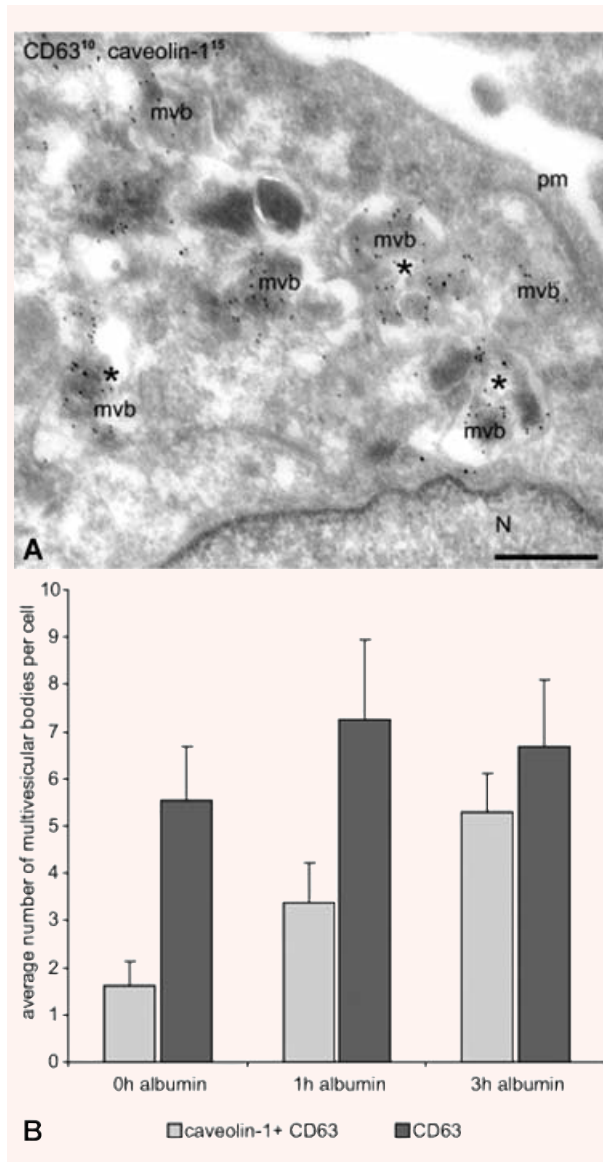


Fig. 5 Albumin incubation increases the number of caveolin-1 containing multi-vesicular bodies. **A:** Ultrathin cryosection of HepG2 cells immunogold labelled for caveolin-1 (15-nm gold) and CD63 (10-nm gold). After 3 hrs of albumin uptake, caveolin-1 and CD63 double-labelled multi-vesicular bodies (mvb*) appeared in the cytoplasm among single CD63 labelled organelles (mvb). N = nucleus. Bar, 200 nm. **B:** Our morphometrical analysis provides quantitative data showing that after 3 hrs, the number of the double labelled multi-vesicular bodies is significantly higher ($P < 0.005$ versus control [0-hr albumin]).

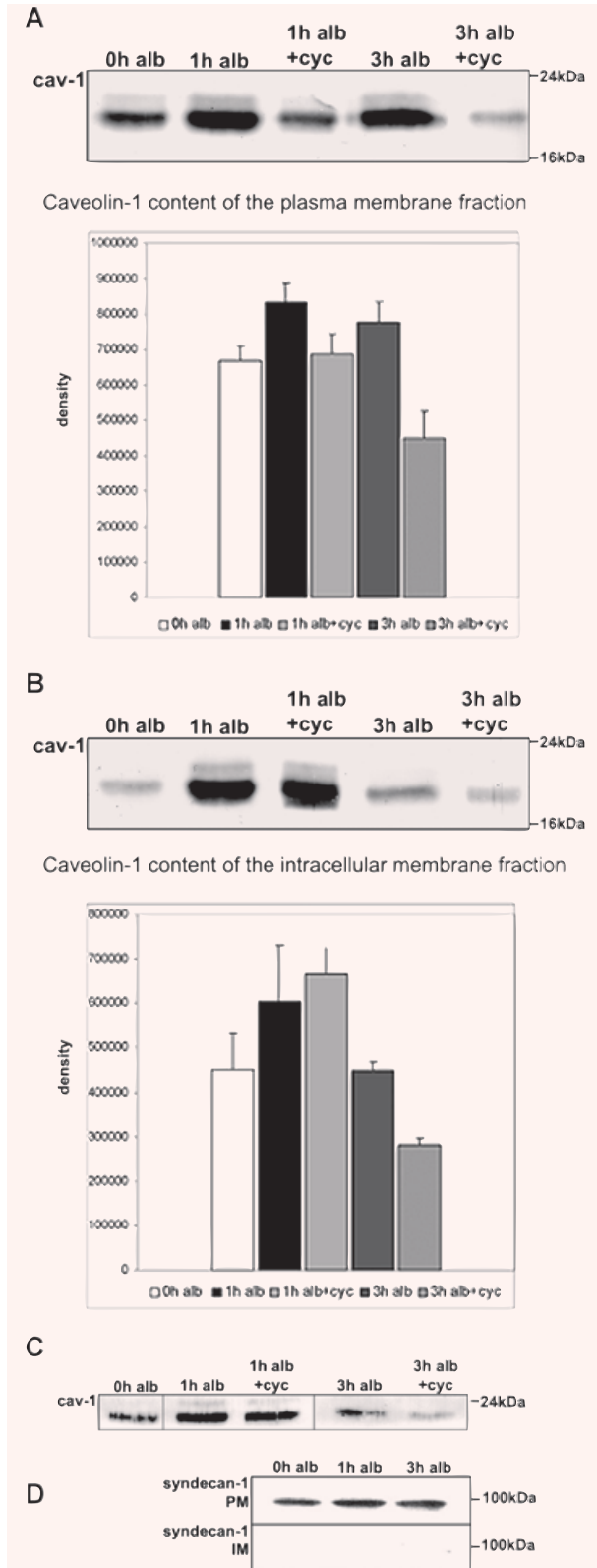
Table 1 Distribution of caveolin-1 on ultrathin frozen sections. Values are expressed as percentage of the total number of gold particles detected above various compartments of the cytoplasm. In non-treated cells (0-min. albumin) more gold particles were counted at the plasma membrane than in internalized vesicles. Albumin treatment resulted in an increased accumulation of gold particles in pinched off caveolae and caveosome-like structures. At the same time, more caveolin-1 appeared in CD63 labelled late endosomes.

	0-min. albumin	60-min. albumin	180-min. albumin
Plasma membrane caveolae	22.6%	18.75%	15%
Pinched off caveolae	13.3%	18.01%	24.5%
Pinched off caveolae in clustered caveolae (caveosomes)	4%	9.1%	5.9%
CD63 labelled late endosomes	30.2%	32.72%	39.73%
Cytoplasm	20%	12.8%	13.24%
Nucleus	5.1%	4.7%	1%
Mitochondria	4%	3.67%	1.3%

(Fig. 5B), whereas after 3-hr albumin, a significant increase in the number of caveolin-1-containing MVBs was found. Colocalization of CD63 and caveolin-1 was restricted to these late endosomes. When gold particles detecting caveolin-1 were counted in different cellular compartments (Table 1), the number of gold particles was found to be larger in caveolae pinched off from the plasma membrane, caveosomes, and CD63-labelled late endosomes by the time of albumin internalization. These data indicate that after prolonged incubation with albumin, caveolin-1 enters the degradative pathway to MVBs and lysosomes.

Distribution of caveolin-1 at plasma membrane and intracellular membrane fractions after albumin incubation: Western blot analysis

To study caveolin-1 distribution during albumin uptake by an additional method, plasma membrane and intracellular membrane fractions were isolated from HepG2 cells by using the detergent-free method of Smart (1995) [15] and Conrad (1995) [16]. The caveolin-1 content of these membranes was analysed by Western blot. These quantitative data showed that in HepG2 cells incubated for 1 hr with albumin, the amount of caveolin-1 present in the plasma membrane increased when compared to albumin depleted (0-hr albumin) cells (Fig. 6A) ($P < 0.005$). This is in agreement with our morphological observations, showing an increase in the



number of caveolae at the plasma membrane upon albumin incubation (Fig. 3). Prolonged incubation, 3-hrs of albumin, did not result in a further increase in the caveolin-1 content of the plasma membrane (Fig. 6A: 3-hrs alb). One-hour albumin uptake also increased the amount of caveolin-1 in the intracellular membranes (including multi-vesicular bodies) (Fig. 6B). However, in contrast with the plasma membrane fraction, this was followed by a decrease in caveolin-1 levels upon longer incubation times (3-hrs albumin). These data are in good agreement with our morphological analysis and suggest that upon albumin incubation, the number of caveolae at the plasma membrane increases, whereas at the same time internalization of caveolin-1 is started, leading to its degradation after 3-hrs incubation.

One would expect that an increased uptake and degradation of caveolae would lead to a depletion of caveolin-1 at the plasma membrane. Instead, we observe an increase, suggesting that degradation of caveolin-1 is accompanied by *de novo* formation of caveolae. To address the question whether the increase in plasma membrane-associated caveolin-1 represents newly synthesized protein, we added 50 µg/ml cycloheximide to our assays to block protein synthesis. Three hours of simultaneous incubation with albumin and cycloheximide results in a significant decrease in the amount of caveolin-1 present in both the plasma membrane (Fig. 6A: 3-hrs alb + cyc), and the intracellular membrane fractions (Fig. 6B: 3-hrs alb + cyc). These data indicate that the increase of caveolae and caveolin-1 levels at the plasma membrane requires *de novo* synthesis.

Discussion

The participation of caveolae in endocytosis was debated for a long time. Nowadays, it is generally accepted that, under special conditions, caveolae can take part in ligand internalization [2].

Fig. 6 Caveolin-1 distribution in different membrane fractions isolated from HepG2 cells. **A:** Albumin-depleted cells present low amounts of caveolin-1 in plasma membrane fraction (0-hr alb). Albumin incubation significantly increases the protein content of this fraction (1-hr alb) ($P < 0.005$ versus control [0-hr albumin]). When albumin was added for 3 hrs, the amount of caveolin-1 was found to be the same as after 1-hr incubation (3-hralb). Three hours of cycloheximide treatment efficiently decreased the caveolin-1 content of the plasma membrane (3-hrs alb + cyc) ($P < 0.005$ versus 3-hrs albumin). **B:** Longer-time (3 hrs) albumin uptake resulted in a significant decrease in the caveolin-1 content of the intracellular membrane fraction (including Golgi membranes, endoplasmic reticulum and membranes of endosomes, lysosomes) (3-hrs alb). **C:** Caveolin-1 content of post-nuclear supernatant (total caveolin-1) under different experimental conditions. **D:** As a marker of the plasma membrane, a transmembrane glycoprotein, syndecan-1 (CD138) was immunoblotted in plasma membrane (pm) and intracellular membrane fractions (IM) isolated from HepG2 cells.

Caveolar endocytosis is a slow, highly regulated process with low capacity. Internalization of caveolae is induced by tyrosine phosphorylation of caveolin-1 and dynamin *via* activation of c-Src kinase [11]. Ocadaic acid, a serine/threonine phosphatase inhibitor, induces caveolar clustering at the plasma membrane, followed by their internalization [17, 18]. Thus, caveolar pinching off is regulated by the synchronized function of kinases and phosphatases [19] and depends on the integrity of the actin-cytoskeleton and presence of dynamin [20].

Although caveolin-dependent endocytosis has been in the centre of interest for a long time, the intracellular compartments involved in this alternative pathway have remained elusive. Caveosomes accumulating internalized ligands like viruses and cholera toxin [6] are considered as intermediate organelles of the caveolar pathway. Ligands endocytosed by clathrin-coated pits are not detected in caveosomes [2]. Caveosomes are not acidic and they do not contain markers of endosomes, lysosomes or endoplasmic reticulum. Until now, the only established marker of caveosomes is caveolin-1. There are a few indications about the possible function and the further fate of caveosomes [2]. One theory postulates that caveosomes could provide an intracellular site where ligands can escape from lysosomal degradation [2]. Alternatively, caveolae can recycle back to the plasma membrane from caveosomes [8]. Internalized caveolae can also fuse with early endosomes in a Rab5-dependent manner [7, 8]. However, it is not known whether caveolin-1 can be degraded by following the classical degradative pathway.

In our work, we were especially interested in the intracellular route of caveolin-1. In order to follow caveolin-1 trafficking after endocytosis, we performed quantitative morphological and biochemical analyses during caveolin-dependent endocytosis of albumin in HepG2 cells. We applied long-term (1 and 3 hrs) albumin incubation to induce internalization of caveolae. We found that the number of caveolae along the plasma membrane strongly depends on the presence of albumin. Long-term albumin treatment resulted in the appearance of numerous caveolae at the plasma membrane with an increased amount of caveolin-1, suggesting that albumin triggers the formation of new caveolae at the plasma membrane. At the same time, the number of internalized caveolae-containing albumin had also increased. Moreover, in addition to numerous single caveolae, caveosome-like structures appeared. These data support that caveolar vesicles follow an intracellular route distinct from the classical endocytotic pathway. Some of these multi-caveolar clusters were seen in connection with the cell surface at the plane of the section. Others were found deeper in the cytoplasm, at a distance from the plasma membrane,

suggesting that they are detached to form an independent structure. Although there are data showing that caveosomes are connected to the plasma membrane [21, 8], it is still debated that caveolar clusters are detaching from the plasma membrane forming caveosomes, or single caveolae can directly leave these caveolar clusters to enter different endocytotic pathways.

Notably, after 1–3-hr albumin incubation, caveolin-1 was also present in multi-vesicular bodies as defined by multiple intraluminal vesicles and presence of CD63. Albumin internalization significantly increased the number of caveolin-1 and CD63 containing multi-vesicular bodies. Our Western blot analysis showed that albumin uptake resulted in an initial increase (1-hr albumin incubation) followed by a significant decrease (3-hrs albumin incubation) of caveolin-1 in the intracellular membranes (including multi-vesicular bodies). These data together indicate that endocytosed caveolin-1 is degraded in multi-vesicular bodies.

In seeming contradiction with the degradation of internalized caveolin-1, we found that the number of caveolae and the caveolin-1 content of the plasma membrane increased. This raised the question whether caveolin-1 is replaced by newly synthesized protein. Indeed, when protein synthesis was blocked by cycloheximide [22] and the cells were triggered to internalize albumin, the amount of caveolin-1 in the plasma membrane is significantly decreased. As a result of degradation and absence of protein synthesis, caveolin-1 present in the intracellular fraction also decreased. These data suggest that internalized caveolae travel to late endosomes, where caveolin-1 is degraded. At the same time, caveolae at the plasma membrane are replenished, which requires *de novo* synthesis of caveolar components.

Our results indicate that distinct triggers could initiate different internalization pathways (*i.e.* degradation or recycling or storage). The regulation might depend on the interaction between the ligand and caveolar components. Caveolae-mediated endocytosis of albumin in HepG2 cells may also result in increased caveolae trafficking as well as *de novo* caveolae formation.

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References

1. **Anderson RGW.** The caveolae membrane system. *Annu Rev Biochem.* 1998; 67: 199–225.
2. **Pelkmans L, Helenius A.** Endocytosis *via* caveolae. *Traffic.* 2002; 3: 311–20.
3. **Krajewska WM, Maslowska I.** Caveolins: structure and function in signal transduction. *Cell Mol Biol Lett.* 2004; 9: 195–220.
4. **Stahlhut M, van Deurs B.** Identification of filamin as a novel ligand for caveolin-1: evidence for the organization of caveolin-1 associated membrane domains by the actin cytoskeleton. *Mol Biol Cell.* 2000; 11: 325–37.
5. **van Deurs B, Roepstorff K, Hommelgaard AM, Sandvig K.** Caveolae: anchored,

- multifunctional platforms of the lipid ocean. *Trends Cell Biol.* 2003; 13: 92–100.
6. **Pelkmans L, Krtenbeck J, Helenius A.** Caveolar endocytosis of simian virus 40 reveals a new two-step vesicular transport pathway to the ER. *Nat Cell Biol.* 2001; 3: 473–83.
 7. **Pelkmans L, Bürlí T, Zerial M, Helenius A.** Caveolin-stabilized membrane domains as multifunctional transport and sorting devices in endocytic membrane traffic. *Cell.* 2004; 118: 767–80.
 8. **Parton RG, Simons K.** The multiple faces of caveolae. *Nat Rev.* 2007; 8: 185–94.
 9. **Schnitzer JE, Oh P, McIntosh DP.** Role of GTP hydrolysis in fission of caveolae directly from plasma membranes. *Science.* 1996; 274: 239–42.
 10. **Minshall RD, Sessa WC, Stan RV, Anderson RGW, Malik AB.** Caveolin regulation of endothelial function. *Am J Physiol Lung Cell Mol Physiol.* 2003; 285: 1179–83.
 11. **Shajahan AN, Timblin BK, Sandoval R, Tiruppathi C, Malik AB, Minshall RM.** Role of Src-induced dynamin-2 phosphorylation in caveolae-mediated endocytosis in endothelial cells. *J Biol Chem.* 2004; 279: 20392–400.
 12. **Slot JW, Geuze HJ, Gigengack S, Lienhard GE, James DE.** Immuno-localization of the insulin regulatable glucose transporter in brown adipose tissue of the rat. *J Cell Biol.* 1991; 113: 123–35.
 13. **Liou W, Geuze HJ, Slot JW.** Improving structural integrity of cryosections for immunogold labeling. *Histochem Cell Biol.* 1996; 106: 41–58.
 14. **Weibel ER, Kiseler GS, Scherle WF.** Practical stereological methods for morphometric cytology. *J Cell Biol.* 1966; 30: 23–38.
 15. **Smart EJ, Ying YS, Mineo C, Anderson RGW.** A detergent-free method for purifying caveolae membrane from tissue culture cells. *Proc Natl Acad Sci USA.* 1995; 92: 10104–8.
 16. **Conrad P, Smart EJ, Ying YS, Anderson RGW, Bloom GS.** Caveola cycles between plasma membrane caveolae and Golgi complex by microtubule dependent and microtubule independent steps. *J Cell Biol.* 1995; 131: 1421–33.
 17. **Eskelinen EL, Tanaka Y, Saftig P.** At the acidic edge: emerging functions for lysosomal membrane proteins. *Trends Cell Biol.* 2003; 13: 137–45.
 17. **Parton RG, Joggers B, Simons K.** Regulated internalization of caveolae. *J Cell Biol.* 1994; 127: 1199–215.
 18. **Kiss AL, Botos E, Turi Á, Müllner N.** Ocadaic acid treatment causes tyrosine phosphorylation of caveolin-2 and induces internalisation of caveolae in rat peritoneal macrophages. *Micron.* 2004; 35: 707–15.
 19. **Pelkmans L, Fava E, Grabner H, Hannus M, Habermann B, Krausz E, Zerial M.** Genome-wide analysis of human kinases in clathrin- and caveolae/raft-mediated endocytosis. *Nature.* 2005; 436: 78–86.
 20. **Pelkmans L, Pürterer D, Helenius A.** Local actin polymerization and dynamin recruitment in SV40-induced internalization of caveolae. *Science.* 2002; 296: 535–9.
 21. **Kirkham M, Fujita A, Chadda R, Nixon SJ, Kurzchalia TV, Sharma DK, Pagano RE, Hancock JF, Mayor S, Parton RG.** Ultrastructural identification of uncoated caveolin independent early endocytic vehicles. *J Cell Biol.* 2005; 168: 465–76.
 22. **Lusska A, Wu L, Whitlock JP Jr.** Superinduction of CYP1A1 transcription by cycloheximide. Role of the DNA binding site for the liganded Ah receptor. *J Biol Chem.* 1992; 267: 15146–51.