The scaffolding domain of caveolin 2 is responsible for its Golgi localization in Caco-2 cells

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Accepted 28 August 2002
Journal of Cell Science 115, 4457-4467 © 2002 The Company of Biologists Ltd
doi:10.1242/jcs.00130

Summary

In this work, we showed that in Caco-2 cells, a polarized cell line derived from human colon cancer that does not express caveolin 1 (Cav-1), there was no detectable expression of caveolin 2 (Cav-2). When Cav-2 was re-introduced in these cells, it accumulated in the Golgi complex. A chimera, in which the scaffolding domain of Cav-1 was replaced by the one from Cav-2, induced a prominent Golgi staining of Cav-1, strongly indicating that this domain was responsible for the accumulation of Cav-2 in the Golgi complex. Cav-2 was able to interact with Cav-1 in the Golgi complex but this interaction was not sufficient to export it from this compartment. Several chimeras between Cav-1 and 2 were used to show that surface expression of caveolin was necessary but not sufficient to promote caveolae formation. Interestingly, levels of incorporation of the chimeras into Triton insoluble rafts correlated with their ability to trigger caveolae formation raising the possibility that a critical concentration of caveolins to discrete domains of the plasma membrane might be necessary for caveolae formation.

Key words: Caveolin, Epithelia, Intestine, Golgi

Introduction

Originally defined as non-clathrin-coated flask-shaped plasma membrane invaginations (Severs, 1988; Rothberg et al., 1992; Anderson, 1993), caveolae are now considered as a subclass within the operationally defined detergent-resistant fractions rich in specialized glycolipid-based domains (Brown and London, 1998; Harder and Simons, 1997; Simons and Ikonen, 1997). Caveolae membranes contain caveolins (their marker proteins) which bind cholesterol and form complexes with glycosphingolipids (GSLs) and glycosyl phosphatidyl inositol (GPI) anchored proteins (for reviews, see Parton, 1996; Harder and Simons, 1997; Anderson, 1998; Schlegel et al., 1998). Caveolae have been implicated in numerous cellular events, including signal transduction (Sargiacomo et al., 1993; Okamoto et al., 1998; Roy et al., 1999; Smart et al., 1999), calcium homeostasis (Fujimoto, 1993), intracellular cholesterol transport (Field et al., 1998; Fielding et al., 1999; Roy et al., 1999), membrane trafficking, such as endocytosis (Montesano et al., 1982; Gilbert et al., 1999), potocytosis (Anderson et al., 1992) and transcytosis (e.g. Schnitzer et al., 1994), and a variety of human diseases (for a review, see Engelman et al., 1998). Given its presence in apical transport vesicles derived from MDCK cells, caveolin 1 (Cav-1) has also been proposed to play a role in the sorting of apically-destined proteins and glycolipid microdomains in the TGN of epithelial cells (Kurzchalia et al., 1992; Dupree et al., 1993). Indeed, antibodies against Cav-1 can inhibit the apical delivery of influenza hemagglutinin (Scheiffele et al., 1998). Sorting of apical components is now thought to rely on the correct assembly of GSLs, cholesterol, GPI-anchored proteins and other apical proteins in the Golgi complex to create apically destined transport vesicles in MDCK cells (Simons and van Meer, 1988; Simons and Wandinger-Ness, 1990). To refer to these specialized lipid [enriched in GSLs (Brown and Rose, 1992)] and detergent-resistant fractions, a number of different acronyms have been proposed (for reviews, see Simons and Ikonen, 1997; Brown and London, 1998; Jacobson and Dietrich, 1999), or more simply lipid rafts whose existence in vivo has been recently confirmed (Friedrichson and Kurzchalia, 1998; Varma and Mayor, 1998). The exact role of proteins of the caveolin family, however, remains to be understood both in the process of forming and maintaining lipid rafts and in apical sorting events. Cells lacking expression of endogenous Cav-1 have been very useful to uncover some of Cav-1 properties after exogenous expression. For example, Cav-1 expression can promote the formation of caveolae in lymphocytes (Fra et al., 1995), Fischer rat thyroid (FRT) cells (Lipardi et al., 1998) and Caco-2 cells (Vogel et al., 1998) but cannot revert in FRT cells the basolateral polarity of a normally apical GPI-anchored protein, GD1-DAF (Lipardi et al., 1998). The role of Cav-2 remains more elusive since most cells in culture express it. It has been suggested that Cav-2 acts as a co-factor for caveolae formation, regulating their shape and size (Li et al., 1998) and that Cav-1 expression is necessary for Cav-2

localization at the cell surface (Mora et al., 1999; Parolini et al., 1999). Caco-2 cells, which do not produce caveolae or synthesize Cav-1, but do form microdomains (Garcia et al., 1993; Mirre et al., 1996), provide an attractive and well-characterized model system for studying the formation of lipid rafts and caveolae and their potential role in the transport process of apical proteins.

In this work we first showed that Caco-2 cells do not express Cav-2 at detectable levels and used them to study Cav-2 subcellular localization in the presence or the absence of Cav-1. Cav-2 was restricted at steady state to the Golgi complex of Caco-2 cells and this localization is not affected by expression of Cav-1 as opposed to what was shown in two other cells types. Furthermore, using chimeras between Cav-1 and -2, we could identify the molecular determinant responsible for this Golgi restriction as the scaffolding domain of Cav-2. This Golgi retention might be responsible for the low number of caveolae formed at the basolateral surface of Caco-2 cells expressing both caveolins. Another conclusion of this work is that expression of caveolins at the cell surface is necessary but not sufficient to promote caveolae formation and that the level of incorporation of the chimeras into lipid rafts might be regulating the building of caveolae.

Materials and Methods

Cell culture, constructs and transfections

Caco-2 cells were obtained from A. Zweibaum (Villejuif, France) and grown as previously described (Garcia et al., 1993). For experiments, cells were grown on Transwells (Costar Data Packaging, Cambridge, MA) and were used between 10 to 20 days after confluency. Caco-2 cells were transfected using Fugene-6 from Roche Diagnostics GmbH (Mannheim, Germany) with PCDNA3 plasmids containing canine Cav-1 cDNA (hygromycin resistance) or Cav-2 cDNA (neomycin resistance) (Scheiffele et al., 1998). Cav-2 cDNA was modified by the addition of a myc-epitope at the C-terminal part of the protein (Scheiffele et al., 1998) or by adding the eGFP either on the N-terminal part (GFP-cav-2) or the C-terminal part (Cav-2-GFP). After overnight sodium butyrate (10 mM) induction, clones expressing different levels of Cav-1 and/or Cav-2 were selected. Antibiotic (G418 and hygromycin) treatments were stopped at least one week before experiments since hygromycin blocks terminal differentiation of Caco-2 cells (Rodolosse et al., 1998). Chimeras between Cav-1 and Cav-2 were done by PCR and the resulting cDNAs were fully sequenced. The selected clones were expressed in Caco-2 cells using pIRES1 (Clonetech, Palo Alto, CA).

Antibodies

Affinity-purified anti-Cav-1 rabbit polyclonal antibody, N20, directed against Cav-1 N-terminal residues 2-21, and the monoclonal antibody against a Myc epitope (9 E10) were from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal antibody anti-Cav-2 was raised against a synthetic peptide using the residues DFGDLEQLADSGDR of canine Cav-2 (Scheiffele et al., 1998). Mouse monoclonal antibody anti-Cav-2 (C57820, IgG1) was purchased from Transduction Laboratories (Lexington, KY). Mouse monoclonal antibody against antigen 525 (Ag525 mAb), a marker of the basolateral membrane, has been described previously (Le Bivic et al., 1988). Mouse mAb against sucrase-isomaltase (SI) (Beaulieu et al., 1989), a marker of apical membranes, was kindly provided by A. Quaroni (Ithaca, NY). Polyclonal anti-PLAP was from Accurate Chemical and Scientific Corp. (Westbury, NY). Mouse anti-APN has been already described in (Le Bivic et al., 1990) and mouse mAb against Giantin was a kind gift from H. P. Hauri (Basel, Switzerland).

Western blot analysis and immunoprecipitations, flotation and velocity gradients

Western blots were performed as already described in (Mirre et al., 1996) while immunoprecipitations were done as in (Le Bivic et al., 1989). Flotation gradients were prepared as described in (Mirre et al., 1996) and velocity gradients were performed as in (Scheiffele et al., 1998), except that no SDS was added to cell lysates and gradients and that Triton X-100 final concentration was 1%. Aliquots were analyzed by SDS-PAGE and western blotting and quantified with BioImage Quantifier software (Bio-image, Ann Arbor, MI).

Indirect immunofluorescence and laser scanning confocal microscopy (LSCM)

Caco-2 cells were stained for immunofluorescence as described before (Gilbert et al., 1991) using the following antibodies Cav-1, Cav-2, SI, Ag525 and 9E10 at 1:100 dilution, except for Giantin used at 1:500 dilution. Secondary antibodies, i.e., fluorescein isothiocyanate (FITC) anti-mouse IgG or tetramethyl rhodamine (TRITC) conjugated anti-rabbit antibodies were used at a 1:200 dilution (Jackson). Samples were examined with a Zeiss LSM confocal system (Carl Zeiss, Germany) and a Zeiss microscope Axiovert microscope 135M. Confocal images were collected using argon and He-Ne lasers with attenuating filters as excitation sources at 488 nm or 543 nm, for FITC or TRITC, respectively. For simultaneous excitation of FITC and TRITC, a double-banded beam splitter DBSP 488/543 was used. Excitation filters FT 510 nm or LP 560 nm, and emission filters BP 515/565 nm or LP 570 nm were used for separate acquisition of FITC and TRITC signals.

Electron microscopy

Filter-grown confluent Caco-2 cells were processed for electron microscopy according to a method used by (Lipardi et al., 1998) for Fischer rat thyroid (FRT) cells. Cells were rinsed three times with PBS and then fixed for 30-60 minutes at room temperature with 2.5% glutaraldehyde plus 0.1% tannic acid in 0.1 M sodium cacodylate buffer pH 7.3. Cells were then rinsed three times in 0.1 M cacodylate buffer and post-fixed for 30-60 minutes with 1% osmium tetroxide in the same buffer. In some cases, a fixative formula including potassium ferricyanide (K_3Fe (CN)6) to the osmium step was used to enhance membrane contrast and preservation in cultured cells. Following post-fixation, filters were abundantly rinsed with the buffer, cut from the holder, and stained en bloc with 3% uranyl acetate in 50% ethanol (or acetone) for 20-30 minutes. Samples were then dehydrated in a graded series of ethanols (or acetones) and finally embedded in epon 812 (Polysciences, Warington, PA).

Immunogold electron microscopy

Filter-grown confluent Caco-2 cells were thoroughly rinsed with PBS and fixed for 1 hour in 8% paraformaldehyde in PBS. After washing the cells were scrapped off the dishes, collected, infiltrated with 6% gelatin in PBS at 37°C, put on ice and infiltrated with 2.3 M of sucrose in PBS. Samples were then frozen in liquid nitrogen. Ultrathin cryosections were incubated overnight at 4°C with anti-Cav-1 antibody (N20) diluted 1:30 or anti-Cav-2 antibody (mAb 65) diluted 1:20 in 5 to 10% goat serum in PBS. Primary antibodies were revealed using colloidal gold 15 nm or 6 nm-conjugated goat anti-rabbit or anti-mouse IgG respectively in the same buffer. Sections were then fixed rapidly with 2% glutaraldehyde, rinsed in bidistilled water and treated with 0.3% uranyl acetate and 1.8% methyl cellulose in bidistilled water on ice.

Results

Characterization of Caco-2 cells expressing Cav-1 and/or Cav-2

In a previous work, we have shown that Caco-2 cells do not express detectable amounts of Cav-1 (Mirre et al., 1996). Now, using a monoclonal antibody against human Cav-2 (mAb65) or a polyclonal antibody raised against canine Cav-2 (Scheiffele et al., 1998), we could not observe any significant staining on the same cells by immunofluorescence (not shown) and could not detect Cav-2 by western blotting on Caco-2 extracts, while human endothelial cells exhibited a strong immunoreactivity (Fig. 1). In order to study the respective roles of Cav-1 and Cav-2 in caveolae formation, microdomain regulation and apical transport, we expressed canine Cav-1 and/or Cav-2 by stable transfection. Cav-1 expression was detected using the N-20 polyclonal antibody and Cav-2 was detected using an anti-myc antibody 9 E10 (Fig. 7A) or two anti-Cav-2 antibodies (not shown). No cross-reaction was observed between Cav-1 and Cav-2 under our conditions using either mAb65 or a polyclonal antibody raised against canine Cav-2 (Scheiffele et al., 1998). Several clones were selected and the relative amount [compared to Madin-Darby canine kidney (MDCK) cells] of Cav-1 and Cav-2 was estimated in all clones used. The range for Cav-1 went from 4 to 150% and from 20 to 700% for Cav-2 encompassing the levels found in MDCK cells (not shown).

Subcellular localization of Cav-1 and Cav-2

We next sought to determine the subcellular localization of Cav-1 and Cav-2 in transfected Caco-2 cells. Clones expressing either Cav-1, Cav-2 or both were double labeled with monoclonal antibodies against endogenous markers of Caco-2 cells and anti-Cav-1 polyclonal antibody (Fig. 2a,c,e) or anti-Cav-2 polyclonal antibody (Fig. 2b,d,f). In agreement with a previous study (Vogel et al., 1998), exogenous Cav-1 was present on the basolateral membrane where it colocalized with a basolateral antigen (Ag525) (Fig. 2c) and in intracellular compartments comprising the Golgi complex as identified by the marker Giantin (Fig. 2e). No significant colocalization was observed with an apical marker, SI (Fig. 2a) and only a minor population colocalized with transferrin receptor or LAMP1 (not shown). Cav-2 was mostly detected in the Golgi complex

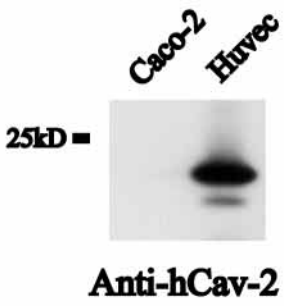

Fig. 1. Analysis of Cav-2 expression in Caco-2 cells. Microsomal fractions of Caco-2 and Huvec (endothelial) cells were analyzed by SDS-PAGE and western blotting. Cav-2 was undetectable in Caco-2 cells but strongly expressed in endothelial cells using an anti-human Cav-2. A molecular mass marker is indicated on the left.

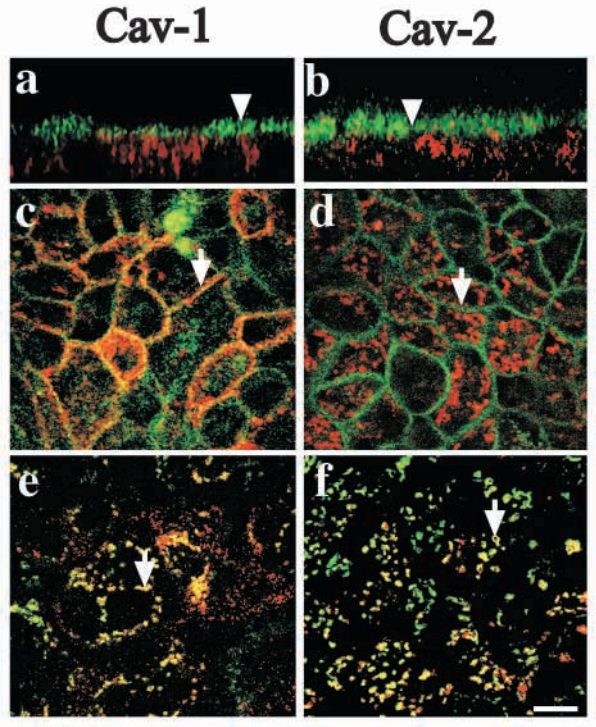

Fig. 2. Subcellular localization of Cav-1 and Cav-2 by confocal analysis of Caco-2 cell clones. Cells expressing Cav-1 (a,c,e) or Cav-2 (b d,f) were double-labeled with rabbit polyclonal anti-Cav-1 (N20) or Cav-2 antibodies (red) and mouse monoclonal antibodies (green) to SI (a,b), Ag 525 (c,d) or Giantin (e,f), used as apical, basolateral or Golgi markers, respectively. Z optical sections (a,b) show that neither Cav-1 (a) nor Cav-2 (b) can be observed at the apical membrane stained for SI (arrowheads). Cav-1 (c,e) is detected both in the Golgi complex and at the lateral membrane (arrows) while Cav-2 (d,f) only showed a perinuclear labeling (arrows). Bar, 5 μm.

where it colocalized with Giantin antibodies (Fig. 2f). No significant overlap was observed with transferrin receptor or LAMP1 by confocal microscopy (not shown). Strikingly, no staining of either apical or basolateral membranes could be detected indicating that Cav-2 did not accumulate at the plasma membrane in transfected Caco-2 cells. This was confirmed by immunoelectron microscopy on frozen sections of Cav-1 and Cav-2 clones (Fig. 3). While gold particles were found on the basolateral membrane with anti-Cav-1 antibodies in Cav-1 cells (Fig. 3B), none were detected in Cav-2 cells using anti-Cav-2 polyclonal antibodies (Fig. 3A). In Caco-2 cells, the Golgi complex was dispersed and very close to the basolateral membrane rather than being concentrated close to the nucleus as in most cells. This was confirmed by electron microscopy in which dictyosomes were seen extending tubules and vesicles within less than 0.5 μm from the lateral membrane (not shown).

Caveolae formation in Cav-1 and/or Cav-2 expressing Caco-2 cells

It has been previously described that exogenous expression of canine Cav-1 in Caco-2 cells promoted the biogenesis of caveolae (Vogel et al., 1998). Since production of caveolae did not correlate with the amounts of exogenously expressed Cav-

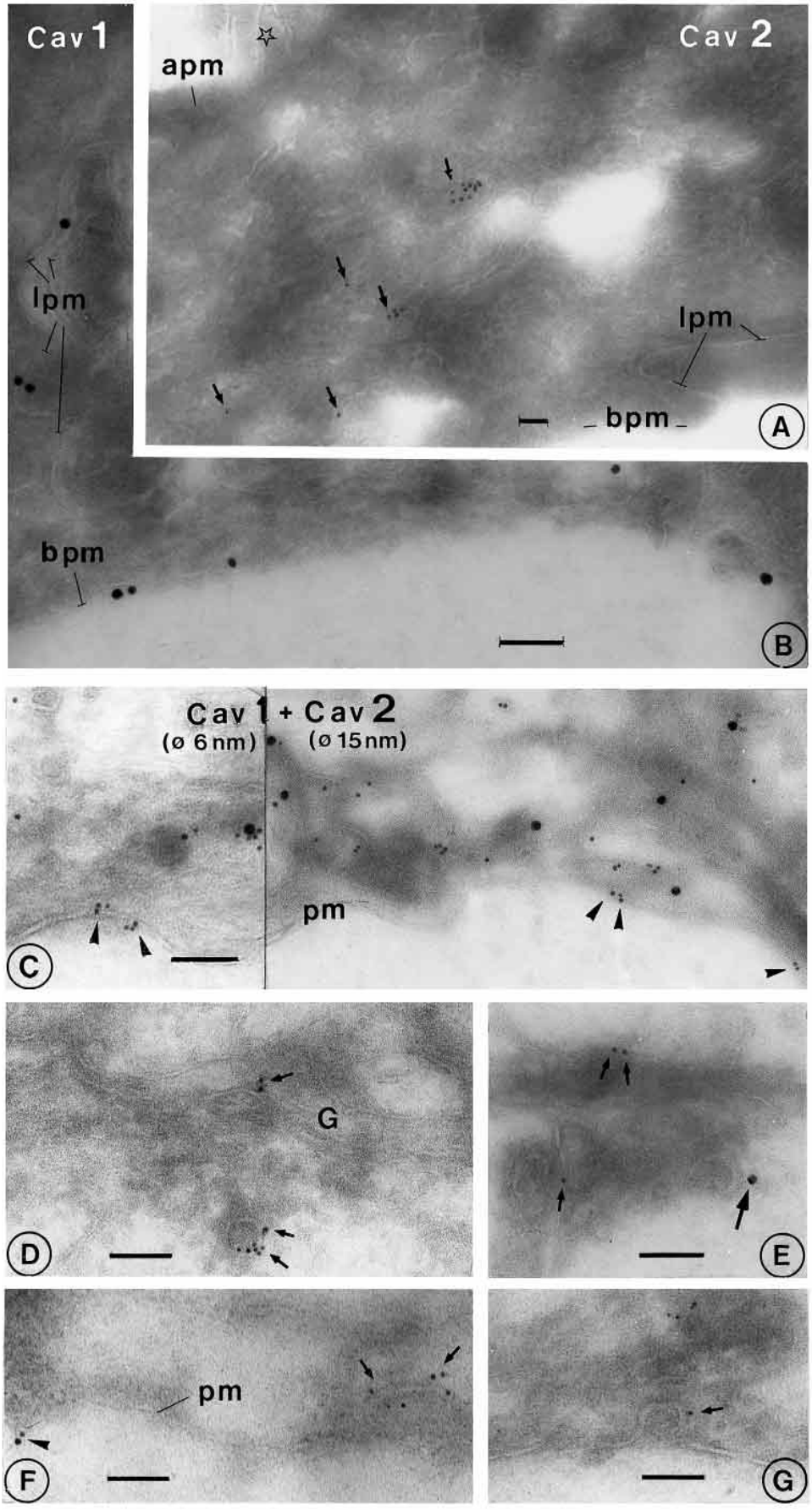

Fig. 3. Immunoelectron microscopic localization of Cav-2 (A), Cav-1 (B) or Cav-1 and Cav-2 (C-G) in transfected Caco-2 cells. Ultrathin cryosections were stained with antibodies to Cav-1 (pAb N20) and/or Cav-2 (mAb65). (A) Cav-2 labeling appeared confined to intracellular structures (arrows). No gold particles were seen at the plasma membrane, neither at the basal (bpm) and lateral (lpm) nor at the apical (apm) plasma membrane. The star indicates apical microvilli. (B) Immunogold labeling for Cav-1 was observed at the basolateral plasma membrane (bpm, lpm). (C-G) Double immunogold labeling of Cav-1 (6 nm gold) and Cav-2 (15 nm gold) in Caco-2 cells co-expressing Cav-1 and Cav-2. (C) The two types of gold particles for Cav-1 and Cav-2 were found distributed on intracellular profiles, but only Cav-1 labeling was also observed along the plasma membrane (pm, arrowheads). (D-E) Gold particles for Cav-1 (small arrows) or Cav-2 (large arrow) were observed on vesicular or tubular membrane profiles. G, putative Golgi complex. (F-G) Immunogold labeling for Cav-1 could be seen here on the plasma membrane (pm, arrowhead), in particular on caveolae (arrows). Bars, 100 nm.

Table 1. Expression of caveolin-1 and 2 in Caco-2 cells and effect on caveolae biogenesis

Transfectant	Number of caveolae at the basolateral surface/mm of filter	Standard error
Cav-1 Cl 8	46	3.3
Cav-1+2 Cl 3	58	9
CH-1	67	6.8

Caco-2 cells expressing Cav-1, Cav-2, both caveolins (Cav-1+2) or CH-1 were processed for electron microscopy and the number of caveolae open to the cell surface was determined on a morphological basis. Whereas almost no invaginated caveolae open to the cell surface were detected in Cav-2 clones (less than 4 mm) or on the apical membrane of all clones, the basolateral membrane of Cav-1, Cav-1+2 and CH-1 exhibited overt caveolae. The number of caveolae is the average of four determinations. No significant difference was observed between caveolae-producing clones.

1, these authors postulated that another factor necessary for caveolae formation was missing in Caco-2 cells. Our finding that Caco-2 cells did not express Cav-2 led us to hypothesize that it could be that factor. We thus quantified caveolae formation in Caco-2 cells expressing either Cav-1 and/or Cav-2 (Table 1). In Cav-1 clones, the average number of caveolae observed by mm of filter was 46, in good agreement with what was described before (Vogel et al., 1998). Cav-2 clones did not show any increase in caveolae production over untransfected cells (less than 4/mm) confirming that Cav-2 by itself was unable to promote caveolae assembly. In cells expressing both caveolins, there was no increase in caveolae numbers over cells expressing only Cav-1 (average 58/mm). Thus expression of Cav-2, in Cav-1-expressing cells, was not able to stimulate caveolae production to the levels observed in MDCK cells (Vogel et al., 1998; Mora et al., 1999). In all clones, caveolae were restricted to the basolateral domain as in MDCK and FRT cells (Vogel et al., 1998; Mora et al., 1999). If Cav-2 acted as a co-factor in caveolae formation, its co-expression with Cav-1 should have increased the number of caveolae formed. Since it was not the case, we looked for the localization of this protein in cells expressing also Cav-1. Co-expression did not alter the typical staining of the Golgi complex and in particular no staining of the plasma membrane was observed (Fig. 4a,b). This data was confirmed by transient expression of Cav-2-GFP fusion proteins with GFP attached either on the N- or C-terminus of Cav-2. Both constructs gave the same intracellular staining in Cav-1 cells with no labeling at the cell surface (Fig. 4c,d). These results were further confirmed by electron microscopy. No labeling of Cav-2 could be detected on the plasma membrane while Cav-1 was found at the plasma membrane and in caveolae-like structures (Fig. 3C,F,G). Internal membranes and vesicles were positive for both caveolins (Fig. 3D,E) indicating that they were indeed in close proximity in the Golgi complex.

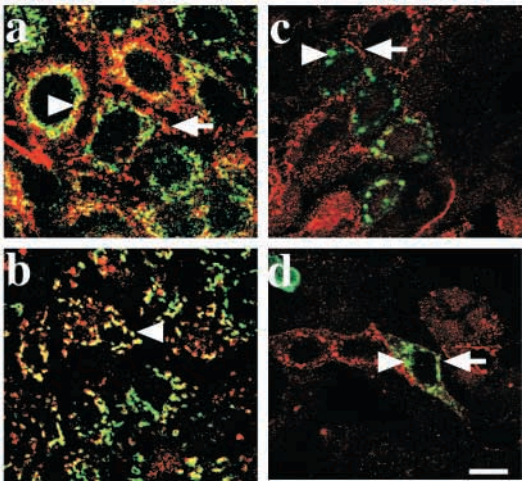

Fig. 4. Localization of Cav-2 in Caco-2 cells expressing Cav-1. (a) Confocal sections showed that Cav-1 and Cav-2 overlapped in a perinuclear region (arrowhead) with Cav-1 showing an additional cytoplasmic and lateral labeling (arrowhead). (b) Cav-2 was still restricted to the Golgi complex co-localizing with Giantin (arrows) in Cav-1 expressing Caco-2 cells. (c,d) Localization of GFP-Cav-2 or Cav-2-GFP (green), respectively, in Cav-1 Caco-2 cells labeled with an antibody against Cav-1 (red). No GFP staining of the plasma membrane could be detected (arrows) while accumulation into an intracellular compartment was observed (arrowheads). Bar, 5 μm.

Cav-1 interacts with Cav-2 without inducing a change in its localization

The lack of Cav-2 redistribution in Cav-1 expressing cells led us to investigate whether the two proteins could interact in Caco-2 cells when co-expressed. In MDCK cells Cav-1 and Cav-2 form oligomers of very high molecular weight as assessed by sucrose velocity gradients (Scheiffele et al., 1998). When expressed alone, Cav-1 was mainly found in the bottom part of such gradients, indicating that it was able to form high molecular weight complexes in Caco-2 cells (Fig. 5A) as in other cells. Cav-2, on the other hand, migrated only in the top fractions of the gradients, suggesting that it was not associated into a complex. When co-expressed, however, a sizeable proportion of Cav-2 (>50%) migrated in the bottom fractions of the gradients together with Cav-1. Thus Cav-1 expression modified the behavior of Cav-2, leading to its inclusion into high molecular weight complexes. In Caco-2 cells expressing both Cav-1 and Cav-2, no Cav-1 was detected in the top-half of the gradient, in contrast with cells only expressing Cav-1, which suggests a possible regulatory effect of Cav-2 on Cav-1 complexes (Fig. 5A). To investigate whether Cav-1 and Cav-2 interact in the same complexes, Caco-2 cells expressing Cav-2 or both caveolins were immunoprecipitated with antibodies against Cav-1 or Cav-2 (Fig. 5B). Cav-2 was able to pull-down Cav-1 from Caco-2 cells expressing both proteins, confirming that the two proteins indeed interact directly or indirectly. Strikingly, Cav-2 was not detected in Cav-1 immunoprecipitates, suggesting that the stoechiometry of the complexes must be in favor of several molecules of Cav-1 for one molecule of Cav-2 as was also reported in MDCK cells (Scheiffele et al., 1998).

The scaffolding domain of Cav-2 is involved in its Golgi localization

In order to understand why Cav-2 is restricted to the Golgi complex while Cav-1 is not, in Caco-2 cells, we produced chimeras between Cav-1 and Cav-2 to identify a region of Cav-

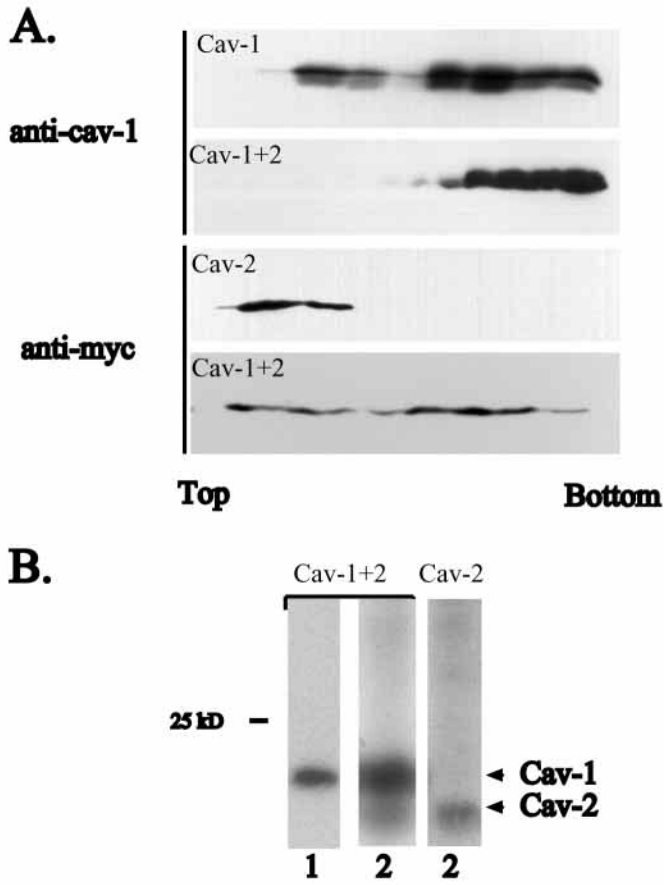

Fig. 5. (A) Cav-1 and Cav-2 form high molecular weight complexes in Caco-2 cells. Caco-2 cells expressing Cav-1, Cav-2 or both, were lyzed, loaded on top of a 5-40% sucrose gradient and centrifuged for 16 hours at 100,000 ***g***. Top to bottom fractions were TCA precipitated, analyzed by SDS-PAGE and immunoblotted with polyclonal caveolin-1 antibodies (anti-Cav-1) or monoclonal anti-myc to detect myc-tagged Cav-2. When expressed alone, Cav-1 was present in high molecular weight complexes while Cav-2 alone was not. Co-expression of Cav-1 along with Cav-2 recruited some Cav-2 and increased the proportion of Cav-1 in high molecular weight complexes. (B) Cav-1 and Cav-2 co-immunoprecipitation in Caco-2 cells. Cav-2 and Cav-1+2 Caco-2 cells were pulse-labeled with ^{35}S methionine for 3 hours and lyzed in 1% Triton X-100. Lysates were immunoprecipitated with polyclonal Cav-1 antibody (1) or mAb65 anti-Cav-2 (2). Immunoprecipitates were analyzed by SDS-PAGE and fluorography. Cav-1 was detected in Cav-2 immunoprecipitates in Caco-2 cells expressing both proteins.

2 that could be responsible for this localization. We designed four chimeras (Fig. 6) which were transfected into Caco-2 cells. Several clones were selected for each chimera and we controlled that the anti-Cav-1 antibody did recognize the chimera produced since it is directed against the first 20 amino acids of Cav-1 (Fig. 7A). All chimeras migrated at a slightly higher molecular position in the SDS-PAGE due to the addition of the myc-epitope. Their subcellular localization was determined using double labeling with endogenous markers and confocal microscopy. Chimeras made of the N-terminal cytoplasmic and transmembrane domains (1 to 152) of Cav-1 (CH-I) or only the N-terminal cytoplasmic domain (1 to 119)

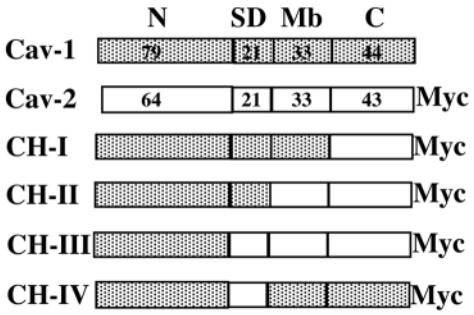

Fig. 6. Cav-1/Cav-2 chimeras. Chimeras (CH-I to IV) between Cav-1 (in shaded boxes) and Cav-2 (open boxes) were made by PCR. A Myc epitope was added at the C-terminal end of Cav-2 and the chimeras. N, amino-domain; SD, scaffolding domain; Mb, membrane domain; C, carboxyl-domain. The numbers of amino acids contained in each domain are indicated.

of Cav-1 (CH-II) were transported to the basolateral surface where they co-localized with the Ag525 while little was found in the Golgi complex (Fig. 7B). Strikingly, when only the first 98 residues of the N-terminal cytoplasmic domain of Cav-1 were grafted to the scaffolding, transmembrane and C-terminal domains of Cav-2 (CH-III), a strong co-localization with Giantin was observed (Fig. 7B). This data indicated that the scaffolding domain (SD) of Cav-2 was responsible for its accumulation in the Golgi apparatus. To test this hypothesis, we designed a chimera in which we replaced the SD of Cav-1 by the one from Cav-2 (Fig. 6). After expression in Caco-2 cells, this chimera containing the SD of Cav-2 (CH-IV) was concentrated in the same compartment as Giantin, demonstrating that the SD of Cav-2 was indeed responsible for its Golgi accumulation (Fig. 7B).

Lipid-raft association but not oligomerization of Cav-1 correlates with caveolae formation in Caco-2 cells

In order to understand the factors controlling caveolae formation in Caco-2 cells, we counted the number of caveolae formed in Caco-2 cells expressing the different chimeras (Table 1). To our surprise, only CH-I was able to promote caveolae formation at levels similar to what was observed with Cav-1. All other chimeras showed numbers of caveolae comparable to untransfected Caco-2 cells (less than 4/mm). Since both CH-I and II were expressed at the cell surface, this condition was necessary but not sufficient to form caveolae. It has been proposed that caveolae formation might be regulated by the ability of Cav-1 to form high molecular weight oligomers. We thus tested the state of oligomerization of the chimeras (Fig. 8B). As opposed to Cav-1 (Fig. 8A), none of the chimeras was able to reach a significant percentage of oligomers and indeed the chimeras behave more like Cav-2 in this respect. Thus, oligomerization and formation of caveolae appeared to be uncoupled events. To try to understand why only CH-I was able to promote caveolae formation, we tested whether the inclusion of caveolins or chimeras into Triton-resistant lipid domains could correlate with the ability to trigger this event. For this the Triton-resistant light membrane (or raft) fractions were obtained after sucrose flotation gradients and analyzed by western blots with the anti-Cav-1 antibody. MDCK cells were

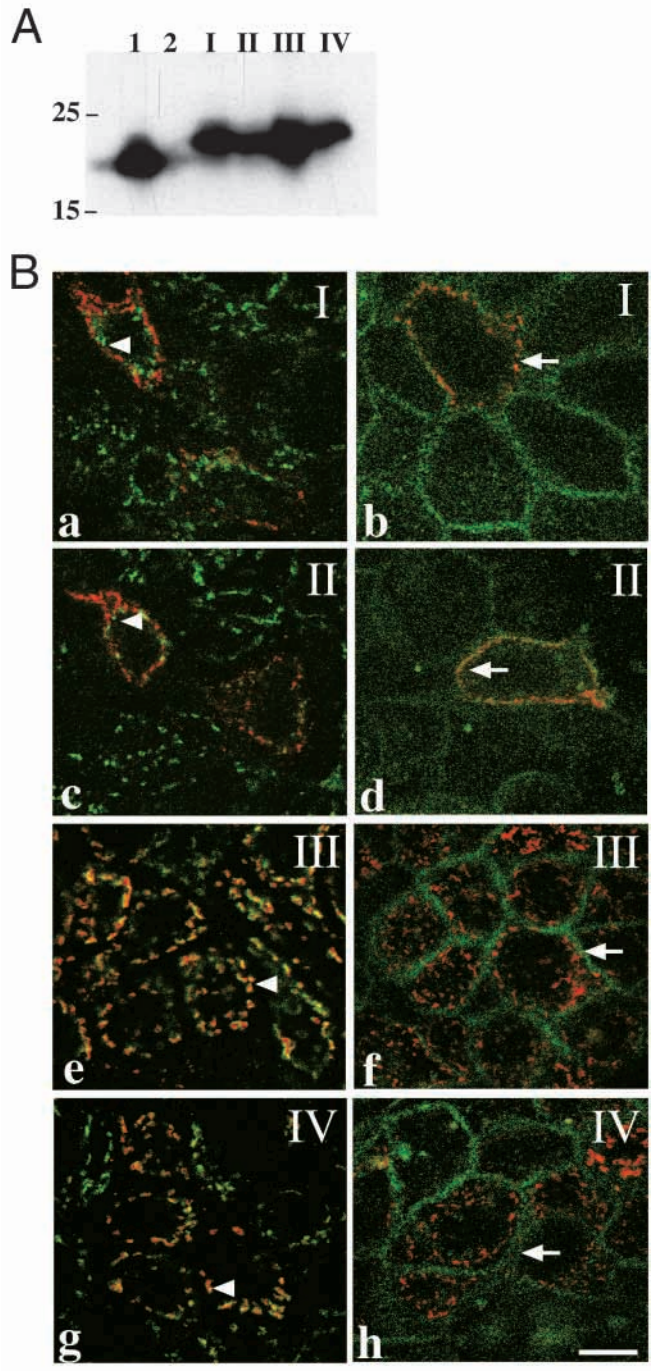

Fig. 7. Expression and localization of chimeras in Caco-2 cells. (A) Stable clones of Caco-2 cells expressing Cav-1 (1), Cav-2 (2) or chimeras I, II, III or IV were lysed and 50 μg of each homogenate was analyzed by SDS-PAGE and immunoblotting with the polyclonal antibody against Cav-1 (N-20). Chimeras I, II, III and IV showed a slower migration because of the addition of the Myc epitope. The molecular mass markers are indicated on the left in kDa. (B) Subcellular localization of chimeras by confocal analysis of Caco-2 cells. Cells expressing Chimeras I, II, III and IV were double-labeled with rabbit polyclonal anti-Cav-1 (N20) (red) and mouse monoclonal antibody (green) against Giantin (a Golgi marker) (a,c,e,g) or Ag525 (an endogenous basolateral marker) (b,d,f,h). CH-I (I) and II (II) can be observed at the periphery of cells marked by arrows while CH-III and IV show colocalization with Giantin indicated by arrowheads. Bar, 5 μm.

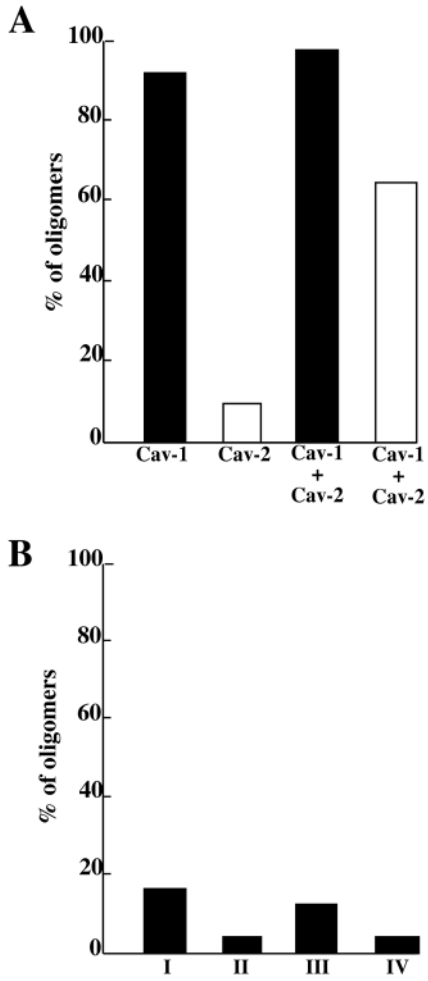

Fig. 8. Oligomerization state of the chimeras in Caco-2 cells. The formation of oligomers by Cav-1, Cav-2 and the chimeras was analyzed as in Fig. 5 and quantified by densitometry after scanning of the western blots using the N-20 (Cav-1 and chimeras) or the anti-Myc antibody (Cav-2). Results are given as a percentage of the amount of protein found in the lower part of the gradients versus the total amount of protein detected into the gradient. (A) Quantification of the percentage of Cav-1 (black bars) and Cav-2 (empty bars) forming high molecular weight oligomers in Cav-1, Cav-2 and double transfected Caco-2 cells (Cav-1 + Cav-2). (B) Quantification of the percentage of chimeras forming high molecular weight oligomers in transfected Caco-2 cells. Cav-2 and the chimeras are mainly found in mono- and dimeric forms as opposed to Cav-1.

used as a control and, in these cells, the majority (60%) of Cav-1 was found in the raft fraction. In Caco-2 cells transfected with Cav-1, this value was 30% and co-expression of Cav-2 raised it up to 50% (Fig. 9A). Cav-2, when expressed alone in Caco-2 cells, was found at low levels (10%) into rafts and co-expression with Cav-1 increased its partition into rafts to 20% (Fig. 9A). Surprisingly, of all the chimeras, only CH-I was predominantly found into rafts (60%) while CH-II, III and IV were found in this fraction at levels that were comparable to Cav-2 (Fig. 9B). These data raised the possibility that there was a relationship between the enrichment of caveolins into rafts and caveolae building.

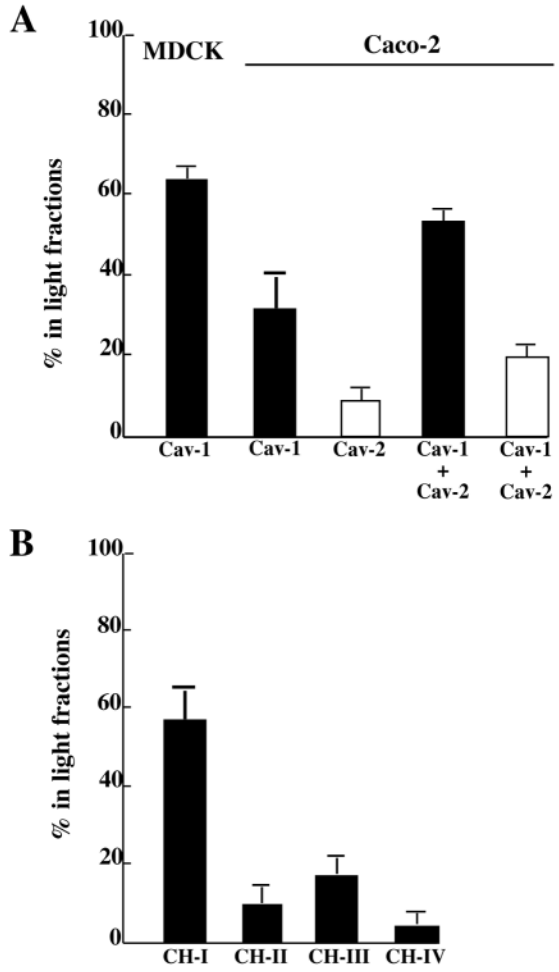

Fig. 9. Association of Chimeras with rafts. (A) MDCK and Caco-2 cells expressing Cav-1, Cav-2 or Cav-1 + Cav-2 were lyzed in a buffer containing Triton X-100 at 4°C and loaded in sucrose gradients. Raft and soluble fractions were harvested and analyzed by SDS-PAGE and immunoblotting with the N-20 antibody for Cav-1 (black bars) or the anti-Myc antibody for Cav-2 (empty bars) in Caco-2 cells. Blots were quantified by densitometry after scanning and the results are expressed as the percentage of protein found in the raft fraction versus the total amount of protein found in the gradient (n=3). (B) Caco-2 cells expressing CH-I to IV were treated as in A using the N-20 antibody. The results are expressed as the percentage of protein found in the raft fraction versus the total amount of protein found in the gradient (n=3). Only CH-I is found in majority in the raft fraction.

Discussion

Cav-2 expression in Caco-2 cells

Using a monoclonal antibody (mAb65) against human Cav-2, we were not able to detect the protein in Caco-2 cells by immunofluorescence and western blotting. With the same antibody, however, human endothelial cells showed a strong reactivity validating its capacity to recognize human Cav-2 under our conditions. We thus conclude that Caco-2 cells express levels of Cav-2 below our detection limits. This is in good agreement with the recent finding that normal colon cells in vivo do not have detectable amounts of Cav-2 (Andoh et al., 2001; Fine et al., 2001). To our knowledge this is the first report of a mammalian cell line that has no detectable protein expression of either Cav-1 (Mirre et al., 1996) or Cav-2 (this work). Several mammalian cell lines have been described that do not express Cav-1 (Shyng et al., 1994; Zurzolo et al., 1994; Fra et al., 1995; Mirre et al., 1996; Parolini et al., 1999). All of them however, either expressed Cav-2 (Mora et al., 1999; Parolini et al., 1999) or were not tested for its expression. There are still conflicting results about the correlation between the levels of Cav-1 and the state of tumorigenesis of colon cells. One group observed an elevated expression of Cav-1 in adenocarcinomas of the colon (Fine et al., 2001) while another group (Bender et al., 2000) reached an opposite conclusion. Thus the exact role of Cav-1 in regulating tumorigenesis has yet to be established. On the other hand, there is no link yet between loss Cav-2 of expression and tumorigenesis. This aspect of Cav-2 possible functions thus needs further investigation. To answer some of the questions raised by previous studies on the potential role of the caveolin family, Caco-2 cells provide a very powerful model to study the actual role of these proteins in cellular functions.

Interactions between Cav-1 and Cav-2

When expressed in Caco-2 cells, Cav-1 and Cav-2 showed different subcellular localization. Cav-1 was found in the Golgi complex, in intracellular vesicles and in the basolateral membrane confirming and extending a previous study performed with an antibody recognizing only plasma membrane Cav-1 (Vogel et al., 1998). In addition we showed that Cav-1 is below detection levels at the apical membrane as opposed to what was observed in MDCK cells. Exogenous Cav-2 was accumulated in the Golgi complex of Caco-2 cells as identified by colocalization with Giantin. No Cav-2 could be detected at the cell surface under our culture conditions suggesting that it might never reach the plasma membrane. Accumulation of Cav-2 in the Golgi complex of FRT and K562 cells has been reported (Mora et al., 1999; Parolini et al., 1999). In both cases, this localization was observed in the absence of Cav-1 expression and Cav-1 transfection induced a partial redistribution of Cav-2 to the cell surface demonstrating that Golgi retention was in part a consequence of a lack of Cav-1. In Caco-2 cells, however, Cav-1 expression did not modify Cav-2 localization suggesting that another limiting factor was missing to export Cav-2 to the plasma membrane. While Cav-1 could not re-localize Cav-2, both proteins could associate with each other since antibodies against Cav-2 immunoprecipitated Cav-1 in co-expressing cells. This association correlated with the ability of Cav-1 to recruit Cav-2 into high molecular weight complexes as it was shown in MDCK, FRT and K562 cells (Scheiffele et al., 1998; Mora et al., 1999; Parolini et al., 1999). On the other hand, Cav-2 seemed to stabilize Cav-1 present in these larger complexes since in its absence Cav-1 was also detected in complexes of lower molecular weight. Formation of Cav-1/Cav-2 complexes might start early when both caveolins exit the endoplasmic reticulum. The localization of Cav-2 in the Golgi complex at steady state implies that Cav-1 might still interacts with Cav-2 in this compartment. This interaction appeared to be rather stable since complexes of Cav-1 and Cav-2 could be co-immunoprecipitated with Cav-2 antibodies even 15 hours after a metabolic pulse (not shown). Whether this interaction needs

to be broken to allow Cav-1 exit from the Golgi complex remains to be investigated. Alternatively, a pool of Cav-1 might never associate with Cav-2 and reach the cell surface as in Cav-1 expressing cells. It is worth to note that a similar situation was recently found in mouse macrophages. In these cells Cav-2 is present primarily in the Golgi complex while Cav-1 is accumulated at the cell surface (Gargalovic and Dory, 2001) suggesting that the two proteins can be uncoupled in cells and that the role of Cav-2 might be more than a simple accessory protein for Cav-1.

Role of the scaffolding domain in the accumulation of Cav-2 in the Golgi complex

To identify the determinants responsible for the accumulation of Cav-2 in the Golgi complex of Caco-2 cells we have chosen to use chimeras between Cav-1 and 2, a strategy respecting the normal conformation of caveolins, which are suspected to have a hairpin structure. Using this approach we demonstrated that the switch of the SD of Cav-1 by the same region from Cav-2 was enough to ensure the accumulation of Cav-1 in the Golgi complex. So far, several studies have been performed to dissect the molecular requirements regulating the subcellular localization of Cav-1 but these studies used a totally different strategy. Instead of chimeras, truncated proteins were produced and transfected to follow their intracellular behavior (Schlegel and Lisanti, 2000; Machleidt et al., 2000; Luetterforst et al., 1999). This approach led to the identification of several regions of Cav-1 that control its subcellular localization or membrane association. In particular, it was found that the C-terminal end of Cav-1 (Schlegel and Lisanti, 2000) or Cav-3 (Luetterforst et al., 1999) was enough to ensure Golgi association. Thus one possibility is that by introducing the SD of Cav-2 into Cav-1, a plasma membrane targeting signal was removed from Cav-1 leading to the preponderance of the Golgi C-terminus signal. It was also demonstrated that part of the SD of Cav-1 appears to control its exit from the Golgi (Machleidt et al., 2000) confirming this hypothesis. Thus, the SD of Cav-2 allows this protein to have a different subcellular localization from Cav-1, strongly suggesting that the two proteins have different fates and probably different partners and functions. This is also supported by the fact that the SD of Cav-1 binds to a consensus sequence in its partners while Cav-2 failed to interact with these proteins under the same conditions (Couet et al., 1997).

Role of Cav-1 and Cav-2 in caveolae formation

Previously, we had shown that Caco-2 cells did not express Cav-1 and did not show any morphologically recognizable caveolae (Mirre et al., 1996). This was confirmed later and in addition it was shown that the expression of canine Cav-1 in these cells led to caveolae formation (Vogel et al., 1998). Surprisingly, not only the number of caveolae produced was 10 times lower than in MDCK cells but, it was also independent of the levels of Cav-1 expressed whereas, in lymphocytes, a correlation between Cav-1 expression levels and the number of newly generated caveolae was observed (Fra et al., 1995). We hypothesized that the lack of Cav-2 expression could have been the reason why caveolae formation was not optimal in Caco-2 cells. However, our data demonstrated that Cav-2 was not able to trigger or stimulate caveolae formation. Cav-2 expression did not increase significantly the number of caveolae in Caco-2 cells that expressed levels of Cav-1 equivalent to MDCK cells. Intriguingly, these results are different from those described in FRT cells (Mora et al., 1999) which only express Cav-2. Expression of Cav-1 in these cells promoted caveolae formation (Lipardi et al., 1998) to numbers roughly similar to those found for MDCK cells (Mora et al., 1999). A likely explanation is that in FRT cells, the expression of Cav-1 induced a partial relocation of Cav-2 to the cell surface that we did not observe in Caco-2 cells. As a consequence, in Caco-2 cells, Cav-2 cannot play its potential regulatory role on the number of caveolae produced at the cell surface. This hypothesis is at odds with the fact that in Cav-2 deficient mouse there is an intact caveolar membrane system, suggesting that Cav-2 has no regulatory role on the number of caveolae (Razani et al., 2002).

The formation of caveolae is correlated to concentration of caveolin into rafts

The mechanisms regulating the formation of caveolae are still poorly understood but a major factor could be the concentration of caveolin 1 molecules in some regions of the plasma membrane to trigger the building of the caveolae scaffold. How this is achieved is not clear but here, we show that when the concentration of Cav-1 or chimeras reaches a given level (more than 30%) in the floating fraction of sucrose gradients it is correlated to a visible effect on caveolae formation. Thus, it is possible that the enrichment of caveolin molecules into lipid rafts creates a microenvironment that favors the association of these proteins into a coat and thus into the formation of invaginated caveolae. It has been shown that Cav-1 binds directly to cholesterol (Murata et al., 1995) and this could explain the concentration of this protein in caveolae that are rich in cholesterol. There is however no data on the affinity of Cav-2 for cholesterol so far but the fact that it is not highly enriched in rafts in Caco-2 cells seems to indicate that its binding to cholesterol is different from the one of Cav-1. Furthermore a recent study has shown that Cav-2 can be found in lipid droplets while Cav-1 was not in the same conditions, indicating that the two proteins might have different lipid affinity (Fujimoto et al., 2001). Do caveolae invaginate from lipid rafts? There is no answer yet to that question. One hypothesis however, is that caveolin molecules when they reach a local concentration in a given lipid environment can bend the plane of the membrane to give rise to the pear shape that is characteristic of these structures. This hypothesis will be tested in vitro using purified Cav-1 with adequate mixes of lipids.

We are grateful to A. Quaroni and H. P. Hauri for giving us antibodies. We thank C. Moretti and J. P. Chauvin for their help in using the confocal microscope and the electron microscope, respectively, and P. Weber for photographic art. We thank G. Trugnan, O. Collard, M. Breton and K. Simons for stimulating discussions and advice and critical reading of the manuscript. This work was supported by CNRS (UMR 6156), Université de la Méditerranée, Institut de Biologie du Développement de Marseille, Association pour la Recherche sur le Cancer 5709, Procope and Galileo collaborative grants, Association Française de Lutte contre la Mucoviscidose and Mizutani Foundation (to A.L.B). L.B. was supported by a fellowship from La Ligue National contre le Cancer.

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