Biogenesis of caveolae: a structural model for caveolin-induced domain formation

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Accepted 21 December 2005
Journal of Cell Science 119, 787-796 Published by The Company of Biologists 2006
doi:10.1242/jcs.02853

Summary

Caveolae are striking morphological features of the plasma membrane of mammalian cells. Caveolins, the major proteins of caveolae, play a crucial role in the formation of these invaginations of the plasma membrane; however, the precise mechanisms involved are only just starting to be unravelled. Recent studies suggest that caveolae are stable structures first generated in the Golgi complex. Their formation and exit from the Golgi complex is associated with caveolin oligomerisation, acquisition of detergent insolubility, and association with cholesterol. Modelling of caveolin-membrane interactions together with in vitro studies of caveolin peptides are providing new insights into how caveolin-lipid interactions could generate the unique architecture of the caveolar domain.

Key words: Caveolae, Cholesterol, Membrane, Model

Introduction

Caveolae, small uncoated pits in the plasma membrane, are an abundant feature of many highly differentiated mammalian cells, such as adipocytes, endothelial cells and muscle cells (Fig. 1). Although caveolae were described morphologically in the early 1950s (Palade, 1953; Yamada, 1955), only recently have some of their functions been revealed. Studies of mice lacking isoforms of the caveolar protein caveolin suggest roles for these structures in lipid uptake and regulation, transcellular transport in endothelial cells, and tumour suppression (Drab et al., 2001; Razani et al., 2002a; Schubert et al., 2001). Caveolae have also been shown to provide a vehicle for the entry of certain viruses into animal cells (Pelkmans and Helenius, 2002). Other work indicates that mutations in the muscle-specific form of caveolin, caveolin-3, cause muscle disease (Engelman et al., 1998). Despite these advances, the relationship between the unique, highly conserved structure of caveolae and their physiological functions remains unclear.

Here we focus on the mechanisms involved in the formation of caveolae. Caveolae are defined by the presence of caveolins and their unique structure, as viewed by electron microscopy (EM) (see Figs 1 and 2). They are considered to be a specialised, morphologically distinguishable form of lipid raft (Rajendran and Simons, 2005; Simons and Toomre, 2000). As discussed below, cholesterol, a crucial component of lipid rafts, is also a vital structural feature of caveolae. Caveolae represent a fascinating model for examining how membrane proteins interact with lipids, how lipid microdomains are generated, and how lipid-protein interactions and lipid rafts can contribute to membrane morphogenesis. Moreover, insights into the formation of caveolae also have implications for the formation of microdomains in many different membrane systems, as well as for our understanding of disease. For example, the developing transverse tubule system of muscle, a surface-connected network of tubules that penetrate the muscle fibre, is enriched in caveolin-3 (Parton et al., 1997), and numerous mutations in this protein have been discovered in patients suffering from a wide range of muscle diseases (Minetti et al., 1998) (Fig. 3). Understanding the effects of these mutations on the normal function of the protein requires an understanding of the precise molecular mechanisms involved in caveolae formation and function. Conversely, the study of the mutants, which have changes in evolutionarily conserved amino acids, are providing new insights into caveolins and caveolae.

Caveolar structure

Caveolae each comprise a caveolar bulb (approximately 65 nm in diameter) connected to an opening of fairly constant diameter (approximately 45 nm in fast-frozen, freeze-substituted adipocytes) (M. Floetenmeyer, C. Ferguson, B. Marsh and R.G.P., unpublished). In many tissues, multiple caveolae are arranged around a central vacuolar domain. In adipocytes this arrangement is particularly striking and huge surface-connected vacuolar domains are covered in numerous caveolae (Parton et al., 2002) (Fig. 1). In developing muscle fibres, multiple caveolae are connected by a single neck to the plasma membrane, producing large chains of interconnected caveolae (Parton et al., 1997). The mechanisms underlying the formation of these tissue-specific structures are not yet defined, although recent studies suggest that complex rosettes of caveolae are formed when caveolae fuse (Pelkmans and Zerial, 2005). Another structural feature of caveolae in certain endothelia is the presence of a stomatal diaphragm in the neck of the caveolae, which is generated by the protein PV1 (Stan et al., 2004). This fascinating specialisation is discussed elsewhere (reviewed by Stan, 2005).

The caveolar invagination appears to be uncoated (compared with clathrin-coated structures) in many micrographs (Fig. 1) but, under appropriate preparation conditions, a type of coat structure can be visualised by EM. Scanning EM and freeze-

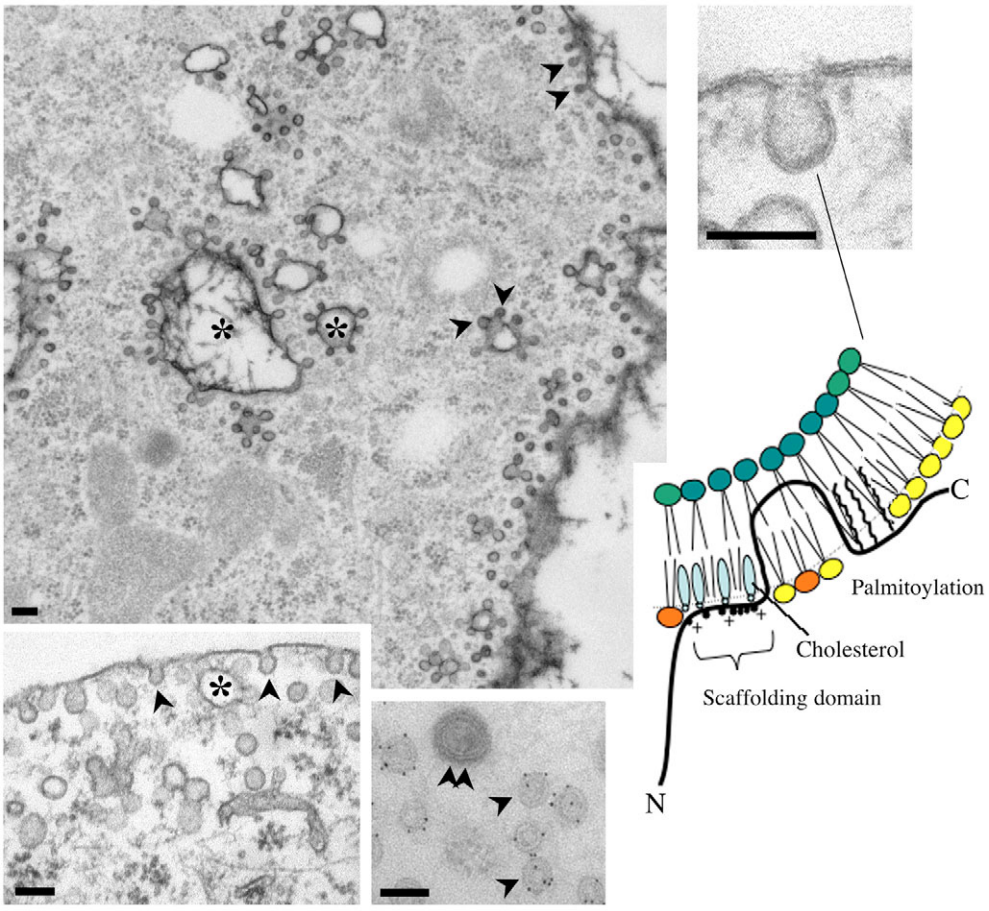

Fig. 1. Caveolae and caveolin structure. Main panel: caveolae in a differentiated 3T3-L1 adipocyte labelled with an electron-dense marker to delineate the cell surface. Note the uniform size and shape of caveolae (some are indicated by arrowheads) and the numerous caveolae around larger surface-connected vacuoles (asterisks). Lower left: caveolae (arrowheads) in a primary fibroblast shown in a conventional plastic section. Top right: a similar image at higher magnification. Lower right: sheets of plasma membrane from adipocytes labelled for caveolin-1 followed by 10 nm protein-A–gold. Note the unlabeled clathrin-coated structure (double arrowheads). The schematic shows the main features of caveolin association with the plasma membrane; positively charged (+) and aromatic (•) amino acids in the scaffolding domain interact strongly with the membrane; schematic modified from Arbuzova et al. (Arbuzova et al., 2000). Cholesterol may be enriched in the scaffolding-domain-associated portion of the membrane. Bars, 100 nm.

etch replica techniques have revealed striations that form a spiral around the cytoplasmic surface of the caveolar invagination (Peters et al., 1985; Rothberg et al., 1992; Stan, 2002). Densities corresponding to these structures are not seen routinely in plastic sections but can be visualised under optimal conditions (see Fig. 2). The molecular composition of this coat remains unknown but it has been proposed that the filaments might comprise oligomers of caveolin (Monier et al., 1995; Peters et al., 1985; Rothberg et al., 1992). Caveolin oligomers can be produced in vitro or purified from tissues (Monier et al., 1995). The oligomers have a distinctive 'necklace' appearance similar to that of the spiral coat. Indeed, purified fragments corresponding to the N-terminal cytoplasmic domain of caveolin-1 (residues 1-101; see Figs 1 and 3) form oligomers that can assemble into filaments (Fernandez et al., 2002) and could correspond to the striated structures of the caveolar coat. Such a model, in which caveolin forms the striations that encircle the caveolar bulb is, however, at variance with the suggestion that caveolin associates only with the neck of the caveolae (Thorn et al., 2003), but is consistent with a recent freeze-fracture study that localised caveolin to a belt around the membrane-proximal region of the caveolar domain of mouse fibroblasts (Westermann et al., 2005).

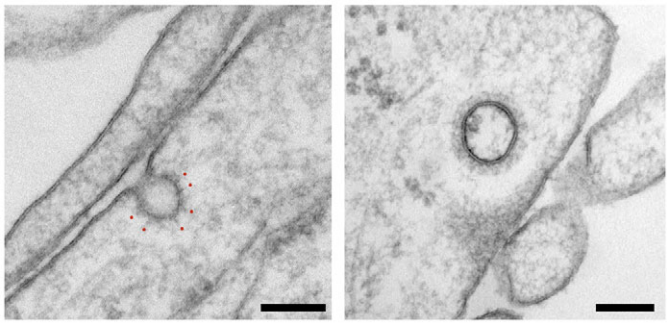

Fig. 2. The caveolae 'coat'. The images show the spiked caveolar coat (left; red dots indicate spikes) as compared with the classical clathrin coat (right) in sections of baby hamster kidney (BHK) cells. The ultrathin plastic sections (<30 nm thickness) allow detection of the coat, which is normally difficult to visualise by conventional EM techniques (compare with Fig. 1). Bars, 100 nm.

The role of caveolins in caveolae formation

The expression of caveolin-1 in cells normally lacking caveolae causes the formation of caveolae (Fra et al., 1995) in various different experimental systems (Breuza et al., 2002; Kirkham et al., 2005; Lipardi et al., 1998; Vogel et al., 1998) and studies showing loss of caveolae in mice lacking caveolin-1 or caveolin-3 confirm the importance of caveolins in this process (Drab et al., 2001; Galbiati et al., 2001). With only a few notable exceptions (see below), caveolin-1 expression correlates qualitatively with the formation of caveolae in non-muscle cells: tissues that exhibit high caveolin expression have a high density of caveolae, whereas those lacking caveolin-1 lack caveolae (Parton, 1996). Both caveolin-1 and the closely

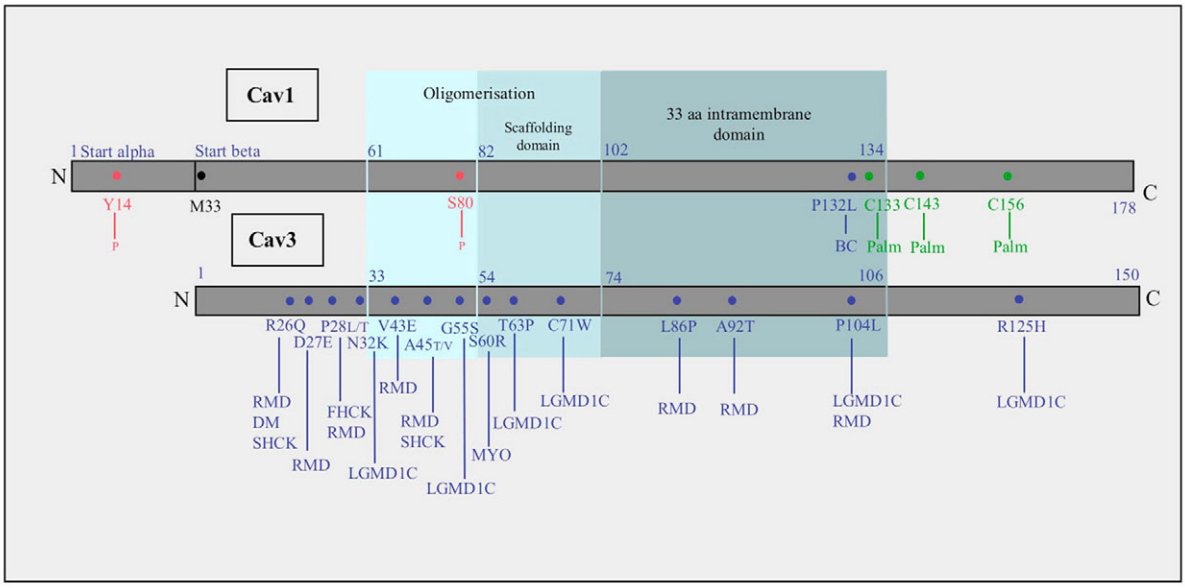

Fig. 3. Sequence features and disease-associated amino acid changes in caveolins-1 (Cav1) and -3 (Cav3). Some key amino acids and features of caveolin-1 and caveolin-3 are shown in relation to defined domains of caveolin-1 and caveolin-3. Numbers above the lines indicate amino acid number in mammalian caveolins. Palmitoylation sites (Palm) in caveolin-1 are indicated in green (potential sites in caveolin-3 are not shown), phosphorylation sites (P) are shown in red, and the starting methionine (M33) of caveolin-1β is shown in black. Caveolin-3 residues are numbered assuming a single methionine residue at the N-terminus. Disease-associated amino acid substitutions in caveolin-1 and caveolin-3 are shown in blue. Abbreviations: RMD, rippling muscle disease; DM, distal myopathy; FHCK, familial hyperCKaemia; SHCK, spontaneous hyperCKaemia; MYO, myopathy; LGMD1C, limb girdle muscular dystrophy 1C; BC, breast cancer.

related isoform caveolin-3 can form caveolae when expressed in cells lacking caveolae (Kirkham et al., 2005). Caveolin-1 is expressed in most non-muscle tissues and in smooth muscle, and is essential for the formation of caveolae in these tissues (Drab et al., 2001). By contrast, loss of caveolin-2, which is generally expressed together with caveolin-1, does not appear to affect formation of caveolae (Razani et al., 2002b) although caveolin-2 might facilitate caveolae formation by caveolin-1 (see below). Caveolin-3 is normally expressed in skeletal muscle, some smooth muscle cells and in cardiac muscle (Tang et al., 1996; Way and Parton, 1995). Caveolin-3-knockout mice have no detectable caveolae in their skeletal and cardiac muscle, which is consistent with caveolin-3 taking the place of caveolin-1 in those tissues (Galbiati et al., 2001). Caveolae formed by heterologous expression of caveolin-1 or caveolin-3 are morphologically identical (M. Kirkham and R.G.P., unpublished; T. Fujimoto, A. Carozzi and R.G.P., unpublished).

Given the studies discussed above and the high concentration of caveolin in caveolae (100-200 caveolin molecules per caveola) (Dupree et al., 1993; Pelkmans and Zerial, 2005), caveolin-1 and caveolin-3 might be structural proteins involved directly in the bending of the membrane to generate caveolae. Alternatively, the shape of the caveolar domain might be generated independently of caveolins but stabilised by caveolin or another protein (e.g. dynamin) (Nabi and Le, 2003).

Caveolin biosynthesis and trafficking

All three caveolins generally behave as integral membrane proteins, although cytosolic and secreted pools of caveolin have also been described (Liu et al., 1999; Uittenbogaard et al., 1998). Caveolins possess a 33-residue central hydrophobic region (the intramembrane domain), which might form a hairpin in the lipid bilayer, flanked by cytoplasmically exposed N- and C-terminal domains (Figs 1, 3, 4). They are synthesised cotranslationally on the rough endoplasmic reticulum (ER) (Monier et al., 1995) and then, in most cells, appear to travel along the secretory pathway to the plasma membrane (Pol et al., 2005); although, see Uittenbogaard et al. for an alternative trafficking model (Uittenbogaard et al., 1998). The transit of caveolins through the Golgi complex is relatively slow compared with that of other membrane proteins (Pol et al., 2005; Ren et al., 2004), and so a Golgi pool of newly synthesised caveolin can be visualised in most cell types (Luetterforst et al., 1999; Nichols, 2002; Pol et al., 2005). Transport of caveolin (but not other integral membrane proteins) through the Golgi complex can be accelerated by cholesterol (Pol et al., 2005). Caveolin-1 is palmitoylated on multiple cysteine residues (Dietzen et al., 1995) in a late-Golgi (BFA-sensitive) compartment (Parat and Fox, 2001) (Figs 1 and 4). This modification is not apparently required for further transport of caveolin-1 or localisation to pre-existing caveolae (Dietzen et al., 1995).

At some stage in the secretory pathway, caveolin changes from a monomeric, detergent-soluble form to an oligomeric, detergent-insoluble form (Pol et al., 2005). The presence of the detergent-insoluble form suggests association with lipid raft domains, although it is important to note that the use of detergents to assess microdomain association has been questioned (Lichtenberg et al., 2005; Munro, 2003). Amino acid substitutions and truncations that disrupt trafficking to the

cell surface cause accumulation of detergent-soluble caveolin in the Golgi complex (Galbiati et al., 1999; Luetterforst et al., 1999; Machleidt et al., 2000; Ren et al., 2004). Rather than revealing cryptic Golgi-targeting or retention sites, these mutations might perturb the caveolin structure, leading to retention of misfolded caveolin in the Golgi complex (Ren et al., 2004). This feature of caveolin mutants is important for understanding certain disease states associated with both caveolin-1 and caveolin-3 (Galbiati et al., 1999). In a recent study, transport of caveolin mutants to the plasma membrane correlated with their ability to associate with lipid raft domains – determined by an in vitro detergent-insolubility assay. Those mutants that accumulate in the Golgi complex generally show reduced raft affinity in this assay (Ren et al., 2004). Thus, incorporation into lipid raft domains in the Golgi complex might be required for formation of caveolae in the Golgi complex or transport of caveolin to the surface prior to their formation.

The site of caveola formation

Where do caveolae form? Transient expression studies and quantitative EM immunolabelling initially indicated that caveolae might form at the plasma membrane by suggesting that a threshold of caveolin is required at the plasma membrane to generate caveolae (Fra et al., 1995). However, more-recent light microscopy studies of the trafficking of newly synthesised caveolin-1–GFP provided evidence for the formation of caveolae in the Golgi complex (Tagawa et al., 2005). In the latter experiments, the intensity of caveolin-1–GFP puncta leaving the Golgi complex is similar to that in surface caveolae, which suggests that 'mature' caveolae are assembled in the Golgi complex. The caveolin-1–GFP-labelled structures apparently fuse directly with the plasma membrane. The caveolin-containing structures may therefore represent carriers in a Golgi-to-plasma-membrane trafficking pathway distinct from those carrying other exocytic markers, such as the vesicular stomatitis virus (VSV) G protein (Tagawa et al., 2005). If so, loss of caveolin-1 might cause a perturbation of this pathway as a result of the absence of the caveolin carriers. This is consistent with the disruption of the post-Golgi transport of specific proteins by caveolin mutants and in cells lacking caveolins (Hernández-Deviez et al., 2006; Wyse et al., 2003).

Under normal conditions, the concentration of newly synthesised endogenous caveolin in the Golgi complex is presumably low [the half-life of caveolin-1 is estimated to be >10 hours (Conrad et al., 1995; Dupree et al., 1993)] but a Golgi pool of newly synthesised caveolin is clearly evident (Dupree et al., 1993; Nichols, 2002; Pol et al., 2005). If formation of caveolar carriers in the Golgi requires a threshold level of caveolin, the slow net transit of endogenous caveolin out of the Golgi complex might allow a sufficiently high level of caveolin to be reached to allow oligomerisation, raft association and formation of carriers. In this model, only fully assembled caveolae would be efficiently transported out of the Golgi complex to the plasma membrane. The characteristics of the Golgi pool of caveolin at steady state [detergent soluble, monomeric (Pol et al., 2005)] would reflect this. Significantly, upon leaving the Golgi complex or reaching the plasma membrane, caveolin can no longer be recognised by certain caveolin antibodies (Pol et al., 2005). Cholesterol depletion restores its reactivity with these antibodies, which suggests a cholesterol-dependent change in caveolin structure on leaving the Golgi complex that would be consistent with the above model.

Once caveolae are formed, they are extremely stable. Fluorescence recovery after photobleaching (FRAP) experiments using

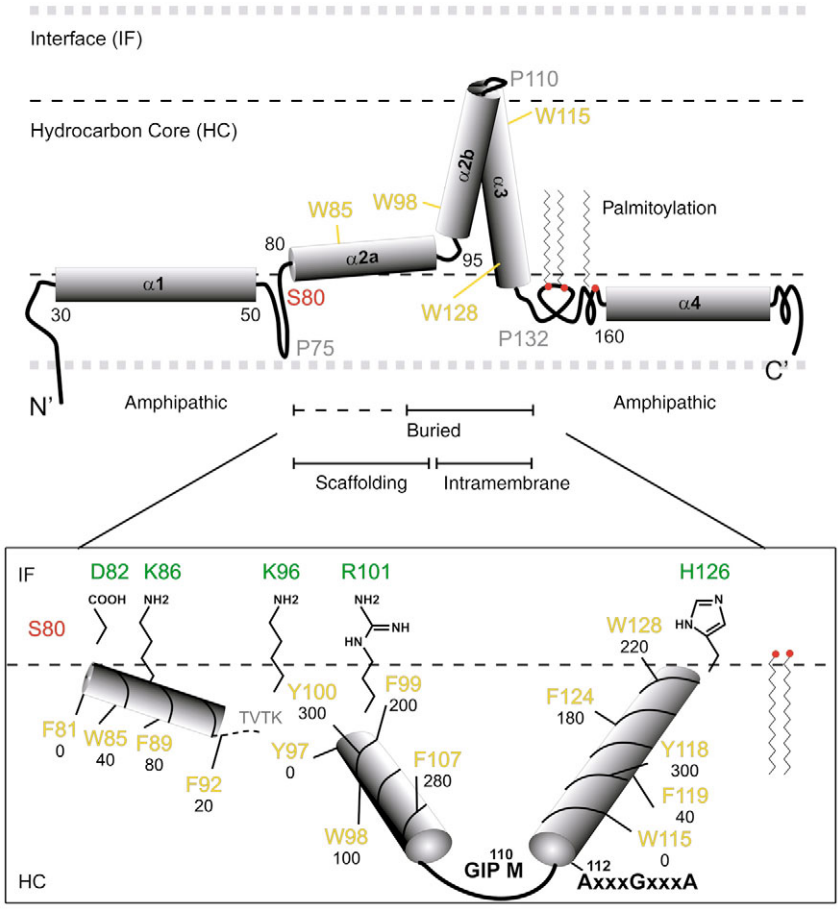

Fig. 4. Topology model of caveolin-1. Helical and buried regions have been predicted from primary structure. The model assumes that charged groups (green) have access to the membrane surface and tryptophan residues (W) are hydrogen bonded. Note that tryptophan residues stay within one helical turn of either the cytosolic (W85, W98, W128) or the exterior (W115) membrane interface. Aromatic residues tend to stay together (black numbers indicate their position in rotational degrees relative to the first aromatic residue of each segment). Segment 80-95 qualifies as an in-plane amphipathic membrane anchor and might compensate for the effect of segment 95-110 being shorter than segment 110-130. Aromatic residues are depicted in yellow, known surface accessible residues in red, and the palmitoyl moieties are to scale. (Numbers correspond to murine caveolin-1.)

caveolin-GFP constructs show that caveolin does not diffuse rapidly in the membrane (Pelkmans et al., 2004; Thomsen et al., 2002) and caveolin molecules within individual caveolae do not exchange with each other (Tagawa et al., 2005). Moreover, real-time experiments have shown that caveolae are relatively immobile. A small fraction can bud; this can be accelerated under certain circumstances – for example, triggering by binding of the virus SV40 or introduction of a phosphatase inhibitor (Kirkham et al., 2005; Pelkmans et al., 2004; Thomsen et al., 2002). Once internalised, caveolae can fuse with a novel endosomal compartment, the caveosome (Pelkmans et al., 2001) or early endosomes (Pelkmans et al., 2004; Sharma et al., 2003; Tran et al., 1987) but remain as discrete units, despite the release of their cargo into the endosomal compartments (Pelkmans et al., 2004). Caveolae can also fuse with the plasma membrane directly in a 'kiss-and-run' cycle (Pelkmans and Zerial, 2005). A lack of surface caveolae in some experiments could therefore reflect a change in the endo/exocytic cycling of caveolae rather than the inability of plasma membrane caveolins to form caveolae.

Additional proteins implicated in the formation of caveolae

Quantitative studies of caveolin expression suggest that additional factors are involved in the biogenesis of caveolae. Caveolin-2 may regulate formation of caveolae driven by caveolin-1, particularly in epithelial cells. In these cells, caveolin-1 is detectable on both surfaces but caveolae generally form only at the basolateral surface. Caveolin-2 is targeted specifically to the basolateral surface (Scheiffele et al., 1998) and interacts with caveolin-1; it could thus be involved in the polarised formation of caveolae at the basolateral surface. Evidence both for and against such a role exists. Overexpression of caveolin-2 in Madin-Darby canine kidney (MDCK) epithelial cells increases formation of caveolae at the basolateral surface (Lahtinen et al., 2003). Furthermore, in a caveolin-negative prostate cancer cell line, caveolin-1 expression is insufficient to generate caveolae but vesicles, assumed to be related to caveolae, accumulate under the plasma membrane (Sowa et al., 2003). Upon co-expression with wild-type caveolin-2, but not caveolin-2 lacking key phosphorylation sites, caveolae are produced. These findings led to a model in which caveolin-2 phosphorylation on Ser23 and Ser36 by casein kinase 2 (or a related kinase) regulates caveola formation. An alternative possibility, which might be compatible with other in vivo and in vitro studies, is that caveolae are very dynamic in this particular cell type. The putative internal caveolae visualised in this study upon caveolin-1 expression might represent budded caveolae, and the lack of surface caveolae might reflect a change in their endo/exocytic cycling. Caveolin-2 might negatively regulate this process in a phosphorylation-dependent manner. With the powerful systems now available to follow budding of caveolae in real time (Pelkmans and Zerial, 2005) and quantitatively assess budding of individual caveolae by EM (Kirkham et al., 2005), this question can now be addressed.

A general role for caveolin-2 in driving caveola formation appears unlikely. Caveolin-1 expressed at levels similar to those observed in naturally expressing caveolin-containing cell lines causes formation of caveolae in the absence of caveolin-2 (Fra et al., 1995). Moreover, caveolin-2-null mice appear to have normal caveolae in the tissues examined (Razani et al., 2002b). Other studies have also shown no effect of caveolin-2 on the formation of caveolae when it is expressed together with caveolin-1 (Breuza et al., 2002) or have shown that caveolin-2 causes formation of more-deeply invaginated caveolae (Fujimoto et al., 2000).

Despite the striking morphology of interconnected caveolae within the developing T-tubule system of muscle (Ishikawa, 1968; Parton et al., 1997), caveolin-3 alone causes formation of caveolae identical to those formed by caveolin-1 when expressed in a non-muscle system (Kirkham et al., 2005) (M. Kirkham and R.G.P., unpublished). Additional, presumably muscle-specific, factors must therefore help form this specialised tubular system during muscle differentiation. Amphiphysin 2/Bin1 (M-Amph2) might provide this role. When expressed in fibroblasts, M-Amph2 generates long surface-connected tubules to which caveolin is recruited (Lee et al., 2002). In developing muscle, it partially colocalises with caveolin-3 and binds to phosphatidylinositol (4,5)-bisphosphate [PtdIns(4,5)P_2], which is dramatically upregulated during muscle differentiation (Lee et al., 2002). Although caveolin-3 is not essential for T-tubule development, the cooperation between caveola-generating caveolin-3 and tubule-generating amphiphysin may be important for the development of the characteristic beaded intermediates involved in the generation of the mature T-tubule (Galbiati et al., 2001; Lee et al., 2002).

The importance of cholesterol

Cholesterol is integral to the formation and maintenance of caveolae. Early studies showed enrichment of sterols at the neck of caveolae (Montesano, 1979). Depletion of cholesterol causes flattening of caveolae (Rothberg et al., 1992; Westermann et al., 2005) and an increase in mobility of caveolin-1–GFP in the plasma membrane (Thomsen et al., 2002). Caveolin binds cholesterol tightly in a 1:1 ratio (Murata et al., 1995), and crosslinking studies using a photoactivatable derivative of cholesterol show that it is a major cholesterol-interacting protein (Thiele et al., 2000). Expression of caveolin in cells lacking caveolae causes enrichment of cholesterol in a low-density, detergent-insoluble floating fraction but the increase is greater than predicted for a 1:1 caveolin-cholesterol interaction (Pike et al., 2002).

The cholesterol-binding domain of caveolin has not yet been pinpointed, but its scaffolding domain (residues 82-101 in caveolin-1; Fig. 3), originally considered to be a protein-interaction domain, is now thought to play a role in membrane interactions. This highly conserved region of caveolin has numerous bulky aromatic residues and key positively charged residues (Figs 1, 4). Interestingly, expression of a dystrophy-associated point mutant of caveolin-3 (C71W) (McNally et al., 1998; de Paula et al., 2001) disrupts Ras signalling in a cholesterol-dependent manner (Carozzi et al., 2002). Substitution of tryptophan, rather than loss of cysteine, is important for this effect (Carozzi et al., 2002), which suggests that an additional tryptophan residue could influence association of cholesterol with caveolae. This is an interesting possibility given a recent in vitro study showing that tryptophan-cholesterol interactions can modulate bilayer curvature (van Duyl et al., 2005). Thus, the interaction of caveolin with cholesterol may be fundamental to the generation

of caveolae. Intriguingly, in a completely unrelated system, a cysteine-to-tryptophan change (but not substitution by other residues) in a lipid-exposed transmembrane segment of the nicotinic acetylcholine receptor dramatically enhances the response of the receptor to cholesterol modulation (Santiago et al., 2001).

The N-terminal end of the scaffolding domain of caveolin-1 contains a conserved serine residue (S80; Fig. 3) that plays a role in regulating cholesterol binding. Phosphorylation of S80 decreases caveolin-1-associated cholesterol whereas a point mutant, S80A, shows increased sterol binding (Fielding et al., 2004). Platelet-derived growth factor (PDGF) stimulates phosphorylation of Y14 upon loss of sterol (Fielding et al., 2004), which suggests that there are complex interactions between different domains of caveolin-1 that link signal transduction to cholesterol binding.

In vitro studies of caveolin-cholesterol interactions have largely focused on the scaffolding domain of caveolin-1 (see Figs 1, 3). The scaffolding domain might at least partially insert into the membrane (Arbuzova et al., 2000) and form an in-plane amphipathic helix with one face exposed (see below). Cholesterol promotes the deeper insertion of the peptides into the membrane but the strongest interaction involves the interfacial region of the membrane. Caveolins contain a potential cholesterol recognition and/or interaction amino acid consensus (CRAC) motif (Li and Papadopoulos, 1998). CRAC motifs have the consensus sequence L/V-(X)(1-5)-Y-(X)(1-5)-R/K- (in which X represents any amino acid) and are present in many proteins that interact with cholesterol (Li and Papadopoulos, 1998). The CRAC motif in caveolin-1 (residues 94 to 101) has not been directly implicated in a simple 1:1 interaction with cholesterol. A comparison of three peptides derived from this region of caveolin-1, one corresponding to the entire scaffolding domain, a second shorter peptide containing the CRAC motif (VTKYWFYR) and a third that does not (KYWFYR) (Epand et al., 2005), shows that the two CRAC-motif-containing peptides associate with liposomes, insert into the membrane and promote segregation of cholesterol into domains (Epand et al., 2005). The sequestered cholesterol is present in crystalline complexes, which indicates that the enrichment is not a result of peptide-cholesterol binding but to an alteration of membrane properties that allows cholesterol enrichment. Indeed, concentration of cholesterol in caveolae is unlikely to reflect a simple 1:1 binding of cholesterol to caveolin because cholesterol is in great excess of any membrane protein.

These studies show that caveolin-cholesterol interactions are complex but that caveolin could cause lateral segregation of cholesterol in the membrane. How this operates in the context of the entire caveolin molecule, which also interacts with the membrane through its intramembrane domain and palmitoylated C-terminus, has yet to be determined. Nevertheless, we can speculate about how these and other regions of caveolin could contribute to caveola formation by drawing comparisons with other membrane-modelling processes.

A model for formation of caveolae

If caveolins are the driving force for formation of caveolae, how does this occur? As described above, one important property of the caveolin-1 and caveolin-3 proteins is their ability to self-associate to form higher-order homo-oligomeric structures. Caveolin-1 forms a 350 kDa complex containing an estimated 14-16 monomers of caveolin-1 (Monier et al., 1996; Sargiacomo et al., 1995). The 41-residue stretch preceding the putative intramembrane domain mediates homo-oligomerisation of caveolin (Sargiacomo et al., 1995). In addition, the C-terminus of caveolin-1 interacts with its N- and C-terminal domains (Song et al., 1997) and this suggested an elegant model for the generation of higher-order oligomeric structures. Oligomer formation would allow formation of discrete membrane domains enriched in caveolin. Whereas oligomerisation is therefore likely to facilitate formation of caveolae, this has not been directly demonstrated and one study concluded that formation of caveolae driven by different caveolin-1–caveolin-2 hybrids correlates with lipid raft association not oligomerisation (Breuza et al., 2002).

How then could caveolin (in the form of monomers or oligomers) modulate membrane curvature? To begin to tackle this question, we have modelled the caveolin-membrane interaction (Fig. 4). Caveolin-1 possesses a central hydrophobic region encompassing both the scaffolding and intramembrane domains (residues 80-130). Its membrane association is likely to be limited on either side by phosphorylation of S80 and palmitoylation of C133 respectively, two processes that probably require access to the cytosol. Using a variety of algorithms, we found that the entire region has high helical probabilities, residues 113-127 (within the intramembrane domain) in particular displaying a plateau (P>0.95) and exceptional hydrophobicity. We could also predict the existence of an additional six-turn helix upstream (α1) between residues 30-50 (P>0.5) that has sharp boundaries and alternates every half turn between buried and solvent-exposed states, which is indicative of an interface helix. The C-terminal residues 160-178 exhibit similar characteristics but lower helical probabilities.

The second half of the hydrophobic region is routinely identified as a transmembrane helix. Using whole-residue hydrophobicity scales (Wimley and White, 1996), we found that residues 110-130 clearly prefer the membrane's hydrocarbon interior to the interface and we assigned them to helix α3. Helix α3 possesses a defined end, showing a sharp drop in helical probability at position P132, a residue that helical C-termini do not tolerate. The complete conservation of P132 in caveolins indicates that this residue might be crucial for a stable structure. Because prolines are present at N-termini of known transmembrane helices, the start of helix α3 is defined by the minimal length necessary to traverse the membrane rather than by residue P110. Our assignment is further supported by the evolutionary conservation of an AxxxGxxxA motif following A112, which resembles the well-known GxxxG motif of transmembrane helices (Russ and Engelman, 2000) and might be required for the close positioning of helices in hairpin structures. Moreover, helix α3 places W115 and W128 within one helical turn of the membrane interface, which is consistent with experimental evidence that tryptophan residues resist translocation into the hydrocarbon core. W115 and W128 are separated by 12 residues, which places their respective Cα atoms ~1.8 nm apart. Taking into account both the length of a hydrogen bond (~0.3 nm) and the distance between Nϵ and Cα within a tryptophan side chain (~0.4 nm), we can conclude that these

12 residues are sufficient to allow both residues to contact lipid head group carboxyl functions in opposing leaflets (~3.2 nm apart). In addition, it has been suggested that tryptophan residues interact with cholesterol by contacting the hydroxyl group and aligning their imidazole moieties with the steroid rings. Thus, in terms of both penetration depth and side-chain orientation, W115 and W128 would be in an ideal position.

Modelling the remainder of the hydrophobic region (the scaffolding domain and first half of the intramembrane domain upstream of α3) is less straightforward. The experimental evidence that the scaffolding domain participates in membrane interaction (Arbuzova et al., 2000; Epand et al., 2005) makes the hydrophobic region much longer than the 33-residue intramembrane domain (102-134) originally proposed, essentially eliminating the need for a hairpin structure. Because a second pair of tryptophan residues (W85 and W98) spaced 12 residues apart is present, one could assign residues 81-101 to a single outbound helix α2 (not shown in Fig. 4). This would raise the interesting possibility that caveolin-2 cannot form caveolae because it lacks the two tryptophan residues in the outer leaflet (W98 in helix α2 and W115 in helix α3). However, we dismiss such a structure because it would connect the two helices through a ten-residue loop (residues 99-109) containing charged residues. This would be highly unfavoured when caveolins localise to lipid droplets because these charges would be placed inside their completely hydrophobic interior (Ostermeyer et al., 2004). Alternatively, if a helical hairpin, i.e. a tight turn connecting two α-helices, were present, it would require turn-forming residues to assume dihedral angles not available to all amino acids. G108 and/or P110 are the only nearby residues that have high turn potential (Monne et al., 1999). However, helical probabilities and hydrophobicity values rise immediately C-terminal of residue S80, which would make helix α2 significantly longer than helix α3. Two helices of 20 residues each would require a very tight turn at a position with low turn propensities, position bulky residues between both helices, and/or place charged residues and tryptophan residues into the hydrocarbon core.

Modelling this 50-residue hydrophobic stretch into a symmetrical structure that does not produce hydrophobic mismatch and yet avoids energetically costly conformations therefore constitutes a major difficulty. One particularly interesting solution to this problem is to orientate region 80-95 (helix α2a) in the plane of the membrane, with all four aromatic residues (F81, W85, F89, F92) pointing into the hydrocarbon core and charged residues (D82 and K86) accessing the cytosol. In this conformation, the diameter of helix α2a in combination with the length of the remaining section (helix α2b) corresponds to the length of helix α3. Interestingly, such in-plane helical membrane anchors have been identified before, and a recent algorithm indicates that the scaffolding domain, which inserts into the interfacial region of the membrane and recruits cholesterol (Arbuzova et al., 2000; Epand et al., 2005), is compatible with such a structure (N. Sapay, Y. Guermeur and G. Deléage, personal communication). We propose that insertion of the scaffolding domain into the membrane is necessary to trigger formation of a caveola. This might occur in the late Golgi, which would be consistent with a cholesterol-dependent conformational 'maturation' step that renders specific caveolin epitopes inaccessible (Pol et al., 2005).

In contrast to the transient recruitment and regulated assembly of coat proteins involved in the formation of clathrin-coated pits and COP-coated buds, the association of caveolin with the caveolar membrane is very stable and clearly quite different. Yet analysis of vesicle formation in these systems and their accessory proteins in particular can provide interesting insights into possible mechanisms of formation of caveolae. Epsin, for example, actually inserts into the bilayer to expand the area of the cytoplasmic leaflet and so facilitates clathrin-coated pit formation (Ford et al., 2002). Because estimates of the number of caveolin molecules per caveola and the size of cytoplasmic–lipid-raft domains suggest that caveolin–lipid-raft microdomains could cover the entire cytoplasmic face of the caveolar bulb, the high local concentration of caveolin in the bilayer might likewise expand the cytoplasmic leaflet. Tryptophan residues at the membrane interface could further increase cholesterol recruitment and insertion of the scaffolding domain into the membrane, achieving greater expansion of the cytoplasmic leaflet of the caveolar bulb.

We have addressed this quantitatively by using standard reference volumes (Harpaz et al., 1994) [refined in Tsai and Gerstein (Tsai and Gerstein, 2002)] to calculate the lipid volume such protein segments would displace (Fig. 5). We first

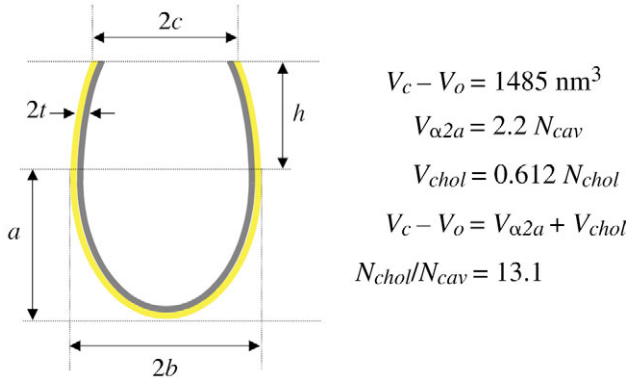

Fig. 5. Model of volume contributions from caveolin and cholesterol in caveolae. The model estimates the increase in volume of the hydrophobic core of the cytoplasmic leaflet (V_c) of the plasma membrane compared with the hydrophobic core of the outer leaflet (V_o) that would be required to cause the curvature characteristic of a caveola. The diagram shows the ellipsoid shape of a fast-frozen adipocyte caveola for which we obtained values of a=42 nm, b=33 nm, c=21 nm, h=29 nm. V_c and V_o were calculated by sequentially subtracting the volumes of a set of three concentric ellipsoids obtained by reducing the dimensions of a, b and c by t and 2t, where t is the thickness of the hydrophobic core of a single leaflet (t=1.5 nm). The volume of a solid ellipsoid of the configuration shown is given by $V=\pi/3\ (2ab^2+2hb^2+hc^2)$. We assume that only the scaffolding domain helix α2a contributes to expansion of V_c because the two membrane-spanning helices (α2b and helix α3) contribute equal volumes to V_c and V_o. We further assume that there are 145 caveolin molecules per caveola (N_{cav}) and that the volume of α2a, $V_{\alpha 2a}$=2.2 nm^3. We propose that the additional volume required to expand the cytoplasmic leaflet (V_c–V_o) is contributed by the ability of the scaffolding domain to penetrate the hydrophobic core of the cytoplasmic leaflet and sequester cholesterol in an asymmetric fashion across the bilayer. N_{chol} is the number of cholesterol molecules sequestered by caveolin, and we assume that the volume of a cholesterol molecule is 0.612 nm^3. In this model, the volume contributed by cholesterol, V_{chol}, would correspond to a molecular ratio of cholesterol to caveolin of 13:1.

calculated the volumes of the inner and outer leaflet of a typical caveolar membrane and compared the relative volume increase of the cytosolic leaflet with the volume contributed by caveolin and cholesterol. We assume that only the scaffolding domain (helix α2a) contributes to expansion of the cytosolic leaflet (V_c) because the two membrane-spanning helices (α2b and helix α3) contribute equal volumes to the inner and outer leaflets of the membrane. These calculations indicate that confinement of the scaffolding domain within the cytosolic leaflet (Fig. 4), together with about 13 molecules of cholesterol per caveolin monomer, could indeed suffice to generate the curvature observed in caveolar invaginations (Fig. 5).

Cholesterol clearly plays an important role in the formation of caveolae but its concentration in the cytoplasmic leaflet of the curved caveolar bulb is energetically less favourable owing to its negative spontaneous curvature (van Duyl et al., 2005). Differences in the lengths of the transmembrane segments of caveolin might therefore be functionally important. For instance, a negative hydrophobic mismatch of helix α2b and/or the insertion of the scaffolding domain as an in-plane helix can generate local membrane deformations that have positive curvature, thus stabilising cholesterol in the vicinity (van Duyl et al., 2005). Alternatively, this could create a curved protein surface, as in the case of amphiphysin, in which membrane curvature is generated through the electrostatic interaction between phospholipids and the concave surface of the BAR (Bin/amphiphysin/Rvs) domain (Peter et al., 2004). Helices of unequal lengths could also produce structural perturbations (Ren et al., 2004) that have effects elsewhere: these could be responsible for the slow net transit out of the Golgi complex, in which caveolin cycling could facilitate caveolin-caveolin and caveolin-cholesterol interactions required for formation of caveolae.

Clearly, many aspects of this model require validation. The absence of structural data on caveolins prevents detailed mechanistic insights at present but the high density of caveolin in the caveolar membrane (Dupree et al., 1993) and the association of caveolin with lipid rafts are certainly consistent with a structural model in which caveolin-cholesterol interactions drive caveolae formation. Our model can also accommodate regulatory mechanisms. For instance, phosphorylation of S80 might cause relocations as a result of changed electrostatics and desolvation energies. The role of this modification and of other specific amino acids can now be tested in relation to the proposed model.

Concluding remarks

Caveolae represent a stable membrane unit built up around caveolin and cholesterol-rich domains in a late Golgi compartment. Caveolin-membrane interactions, and specifically insertion of the scaffolding domain into the membrane and its interaction with cholesterol, might provide the driving force for generation of the unique caveolar structure. This process must also be subject to regulation; recent high-throughput studies of caveolae internalisation implicate kinases in the regulation of caveolin assembly and disassembly (Pelkmans et al., 2005; Pelkmans and Zerial, 2005). Downregulation of the kinase ARAF1, for example, causes loss of caveolin from caveolae and an increase in caveolin mobility. Detailed molecular dissection of these effects is now possible. High-resolution structural analysis of caveolae and in vitro reconstitution of caveolae formation will also provide new insights into the molecular mechanisms involved. This will have general implications for the understanding of membrane morphogenesis as well as providing specific insights into caveola-related disease.

This work was supported by a Program Grant from the NHMRC of Australia and an RO1 grant from the NIH of USA. We would like to thank Charles Ferguson for assistance with electron microscopy, Matthias Floetenmeyer for structural analysis of caveolae, Nicolas Sapay for running a prediction prior to public release, and members of the Parton laboratory for discussions. We are also extremely grateful to Stuart McLaughlin and Josh Zimmerberg for their expert comments on the manuscript.

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