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Caveolae: From Cell Biology to Animal Physiology

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***Abstract*——Among the membrane compartments of a cell, vesicles known as "caveolae" have long defied functional characterization. However, since the identification of a family of proteins termed "caveolins", that form and reside in caveolae, a better understanding has emerged. It is now clear that caveolae do not merely play a singular role in the cell, but are pleiotropic in nature—serving to modulate many cellular functions. The purpose of this review is to explicate what is known about caveolins/caveolae and highlight growing areas of caveolar research.**

I. Introduction

Well before the era of molecular biology, electron microscopists of the 1950s were describing the ultrastructural components of the cell. Among these were 50- to 100-nm invaginations of the plasma membrane referred to as either *caveolae intracellulare*, due to their cave-like appearance, or *plasmalemmal vesicles* (Palade, 1953; Yamada, 1955). It would be approximately forty years before the molecular nature of caveolae could be explored following the identification of its signature protein, caveolin. Since that time, the field of caveolin/caveolae research has blossomed, with caveolae being implicated and demonstrated to be important in a variety of cellular functions including endocytic processes, cholesterol and lipid homeostasis, signal transduction, and tumor suppression. Although caveolae and the caveolins are continuously implicated in an increasing array of cellular processes, it is clear that their physiological roles are vastly different depending on the cell type and organ system examined. For example, their endocytic and vasoregulatory functions likely predominate in the vasculature, whereas they play an important role in the structural integrity of the musculature. In this regard, insights into caveolar function will not only be interesting from the standpoint of cell biology but will be rewarding in understanding mammalian physiology with applications to human disease.

In accordance with increasing knowledge and understanding, the subject of caveolae and the caveolins has been the focus of numerous review articles, with most confined to certain aspects of their function (Parton, 1996; Anderson, 1998; Okamoto et al., 1998; Kurzchalia and Parton, 1999; Smart et al., 1999; Razani et al., 2000b; Schnitzer 2001). Recently, the field has been invigorated by the characterization of caveolin/caveolae-deficient mouse models, thus for the first time enabling investigators to analyze the cellular functions of caveolae with respect to mammalian physiology. As a consequence, it is the purpose of this review to provide a broad overview of the field with detailed discussions on its salient features and to provide a link between the understanding of caveolae at the cellular level and their emerging roles at the organismal level.

II. Caveolae

A. Definition and Morphology

Originally caveolae were given the exclusive electron microscopic description of membrane invaginated "smooth" vesicles of 50 to 100 nm in size (as opposed to the more electron-dense and larger "coated" vesicles—i.e., clathrin-coated pits). However, with further investigation the definition of caveolae has expanded to also include vesicles detached from the plasma membrane proper, associated in groups as grape-like clusters or rosettes, and even in fused form as elongated tubules or *trans*-cellular channels (Fig. 1A). Intriguingly, these "nontraditional" forms of caveolae are more often found

in certain tissues. For example, grape-like clusters of caveolae are highly abundant in developing skeletal muscle cells, with rosettes in adipocytes, and detached vesicles/tubules in endothelial cells (Simionescu et al., 1975; Scherer et al., 1994; Parton et al., 1997). Figure 1B shows an electron micrograph of an adipocyte with the several rosette structures and an endothelial cell with traditional caveolae and detached plasmalemmal vesicles. Therefore, as more is learned about these structures, caveolae can no longer be considered static inpocketings of the plasma membrane, but can take on disparate shapes and forms by conglomerating and/or fusing with one another. The processes involved in the formation of these various caveolar morphologies are largely unknown.

B. Tissue Specificity

At the ultrastructural level, morphologically identifiable caveolae can be found at the plasma membrane of numerous tissues and cell types, albeit in vastly differing abundance. A compilation of morphological data from the past several decades reveals that although most cell types contain some caveolae, certain cells have an extraordinary abundance of caveolae, namely adipocytes, endothelial cells, type I pneumocytes, fibroblasts, smooth muscle cells, and striated muscle cells (Palade, 1953; Napolitano, 1963; Mobley and Eisenberg, 1975; Gabella, 1976; Gil, 1983). It has been reported that in tissues such as the lung up to 70% of the alveolar plasma membrane area (composed of type I pneumocytes and endothelial cells) could be occupied by caveolae (Gil, 1983). However, it should be noted that certain cells are curiously devoid of these invaginations (e.g., central nervous system neurons and lymphocytes) (Fra et al., 1994; Cameron et al., 1997). The reasons for such wide-ranging tissue- and cell-type-specific caveolae expression are not yet known. However, as will be discussed below, the answer might lie in the fact that some of the proposed functions for caveolae would suit certain cell types better than others.

C. Composition and Biochemical Properties

Traditionally, the lipid bilayer of the plasma membrane has been viewed as a two-dimensional "fluid mosaic" (Singer and Nicolson, 1972). In this so-called "liquid-disordered" or "liquid-crystalline" state, the plasma membrane is mostly composed of loosely packed phospholipids capable of rapid lateral diffusion. It has been known for some time, however, that membranes also exist in "liquid-ordered" states, where bilayer assembly is more rigid with confined movement of lipids. Based on a variety of observations, it is now becoming clear that liquid-ordered and -disordered states can coexist on the same plasma membrane and that liquid-ordered domains can remain insular and maintain their relative rigidity among the neighboring phospholipid bilayer. Such domains form via a coalescence of cholesterol and sphingolipids (glycosphingolipids and sphingomyelin)

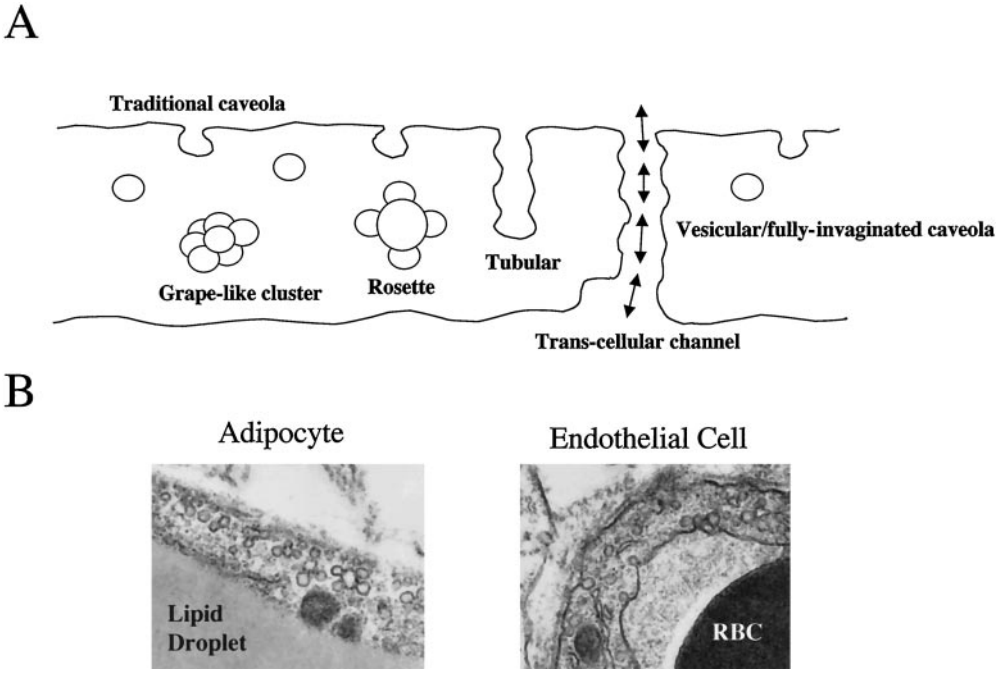

FIG 1. Caveolae: morphological definitions and plasticity. A, caveolae can exist in many alternate forms than the traditional membrane-invaginated variety. They can be found in vesicular form or in aggregates such as grape-like clusters, rosettes, and even elongated tubules. In fact, in endothelial cells, the elongated tubes can sometimes adjoin with the abluminal side, thus creating a trans-cellular channel. B, two electron micrographs, an adipocyte (shown on the left) and an endothelial cell (shown on the right) were taken at a magnification of 16,000×. Caveolar rosettes are frequently observed in adipoctyes, and endothelial cells are often found with caveolae in all states of membrane association—from membrane-bound to fully invaginated.

and thus have been termed *lipid rafts* (Brown and London, 1998; Simons and Toomre, 2000) (Fig. 2A).

Given their shared biochemical properties, caveolae have traditionally been considered a specialized form of lipid raft (i.e., an invaginated/vesicular form) (Fig. 2B) (Brown and London, 1998; Simons and Toomre, 2000). This generalization is probably not entirely accurate as it is now known that certain proteins preferentially partition into lipid rafts or caveolae but not both (Liu et al., 1997); the reader is referred to a more detailed review of lipid rafts (Brown and London, 1998; Simons and Toomre, 2000). The unusual lipid composition of lipid rafts/caveolae imparts particular properties to these microdomains, namely a highly reduced density compared with their phospholipid counterparts and resistance to solubilization by mild nonionic detergents such as Triton X-100 at 4°C. These properties form the basis for the biochemical identification, purification, and characterization of lipid rafts/caveolae (Brown and Rose, 1992; Lisanti et al., 1994b). For example, one of the simplest and most commonly used purification techniques (sucrose gradient ultracentrifugation) utilizes the detergent resistance and buoyancy of these microdomains to separate them from all other cellular constituents (Lisanti et al., 1994b).

Although caveolae and lipid rafts share certain biochemical properties, the localization of the caveolin proteins to caveolae distinguishes these membrane domains. The caveolins serve as selective markers for caveolae (Fig. 2B) and thus allow for the specific analysis of caveolae function.

III. The Caveolin Gene Family

A. The Discovery of Caveolin and Its Relationship to Caveolae

The discovery of caveolin, the original member of the three-protein caveolin family, and its relationship to caveolae was converged upon by investigators from different fields with disparate research interests. In an antibody screen for tyrosine-phosphorylated substrates in Rous sarcoma virus-transformed fibroblasts, Glenney and Zokas (1989) isolated four predominant proteins. Tyrosine phosphorylation of one of these proteins, a 22-kDa molecule, was strongly correlated with the transformation potential of Rous sarcoma virus-transformed cells suggesting a potential role in cellular oncogenesis (Glenney, 1989). Interestingly, antibodies raised against this protein showed punctate staining on the plasma membrane, with a

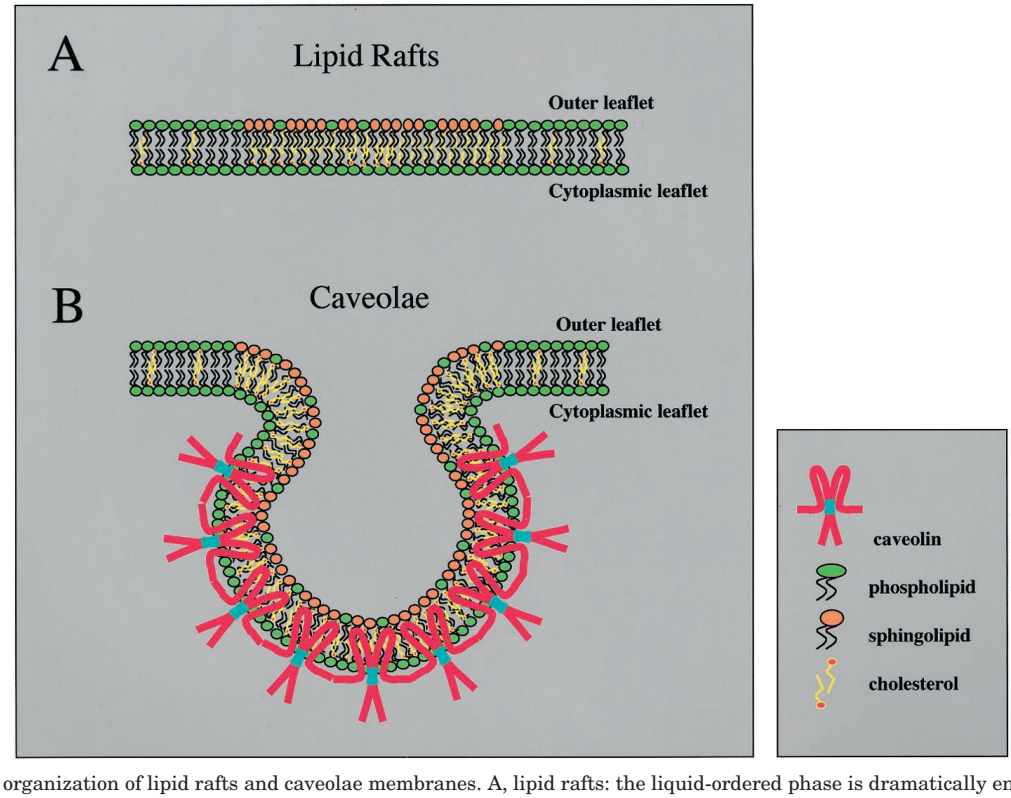

FIG 2. Detailed organization of lipid rafts and caveolae membranes. A, lipid rafts: the liquid-ordered phase is dramatically enriched in cholesterol (shown in yellow) and exoplasmic oriented sphingolipids (sphingomyelin and glycosphingolipids) (shown in orange). In contrast, the liquid-disordered phase is composed essentially of phospholipids, such as phosphatidylcholine, phosphatidylethanolamine, and phosphatidylserine (shown in green). B, caveolae: the liquid-ordered and liquid-disordered phases are illustrated as in panel A. Upon integration of the caveolin-1 protein, liquid-ordered domains form small flask-shaped invaginations called caveolae. Caveolin-1 monomers assemble into discrete homo-oligomers (shown as dimers for simplicity) containing ~14 to 16 individual caveolin molecules. Adjacent homo-oligomers are thought to pack side-by-side within caveolae membranes thereby providing the structural meshwork for caveolae invagination. Caveolin-1 oligomers are red and the caveolin-1 oligomerization domain is shown in blue.

tendency for staining at cellular margins or linear membrane arrays (Glenney and Zokas, 1989; Rothberg et al., 1992). This nonrandom plasma membrane distribution was curiously similar to that observed for the flask-shaped caveolae (i.e., concentrations along the leading edge and the linear stress fibers of cells) and led Rothberg and colleagues to a series of landmark experiments linking this 22-kDa protein and caveolae (Rothberg et al., 1992). The answer was arrived at ultrastructurally when immunogold electron microscopy indicated nearly complete association between the 22-kDa protein and caveolae. More importantly, this protein also seemed to be a major structural component of caveolae. Using rapid-freeze deep-etch techniques, caveolae were shown to be composed of a series of concentric striated rings that stained with antibodies directed against the 22-kDa protein. Moreover, treatment of cells with cholesterol binding agents, such as nystatin and filipin, had profound effects on caveolar morphology, leading to flattening of the vesicles and dissociation of the 22-kDa protein-rich striations. Because this protein was so intimately associated with the structural components of caveolae, it was named *caveolin*, the first bona fide marker of caveolae microdomains (Rothberg et al., 1992).

The subsequent cloning of the caveolin gene led to yet another surprise about the possible functions of the protein (Glenney, 1992; Glenney and Soppet, 1992). In an attempt to identify the cellular machinery involved in the differential sorting of vesicles to the apical or basolateral surface of polarized epithelial cells, Simons and colleagues had cloned VIP-21[1] (vesicular integral protein of 21 kDa), an integral membrane protein component of *trans*-Golgi-derived transport vesicles (Kurzchalia et al., 1992). As it turned out, the caveolin sequence was identical to that of VIP-21, thereby showing that the same protein could possibly serve as a structural component of plasma membrane caveolae, as well as have roles in oncogenesis and vesicular trafficking—all at the same time (Glenney 1992). This series of discoveries marked the beginning of the past decade's investigations into the intimate role between caveolae and caveolin and their intricate functions, topics that we will in turn discuss below.

B. The Other Caveolins (Caveolin-2 and -3)

Since the initial characterization of caveolin, two additional caveolin gene family members have been discovered (caveolin-2 and -3); thus, the original caveolin protein is now known as caveolin-1 (Cav-1) (Way and Parton, 1995; Scherer et al., 1996; Tang et al., 1996). Caveolin-2 (Cav-2) was cloned in an effort to identify other novel resident proteins of adipocyte caveolae (where there is an abundance of caveolae and caveolin-1); caveolin-3 (Cav-3) was found by classic cDNA library screening for Cav-1 homologous genes. In addition to the full-length proteins (α-isoform), both caveolin-1 and -2 have other smaller sized isoforms. The β-isoform of Cav-1 is derived from an alternative translational start site that creates a protein truncated by 32 amino acids (Scherer et al., 1995). The β- and γ-isoforms of Cav-2 have not been analyzed in detail.

An alignment of the human caveolin sequences and an overview of the three caveolin proteins is provided in Fig. 3 and Table 1. There is a relatively high degree of identity between Cav-1 and -3, whereas Cav-2 is by far the most divergent of the three proteins. The highest degree of identity among all three caveolins in the majority of species examined is a stretch of amino acids "FEDVIAEP" and is now referred to as the "caveolin signature motif". The structural/functional significance of this motif remains unknown. Other important domains in the caveolin sequences are the membrane-spanning, oligomerization, and scaffolding domains, all of which will be discussed below (Fig. 3).

All three proteins are present at their highest levels in terminally differentiated cells. As all caveolins are classically known as markers of caveolae, it is not surprising that tissues expressing caveolin-1, -2, and/or -3 are also abundant in caveolae. Interestingly, the expression pattern of caveolin-1 and -2 are largely distinct from that of caveolin-3. Adipocytes, endothelial cells, pneumocytes, and fibroblasts have the highest levels of caveolin-1 and -2, whereas caveolin-3 expression is limited to muscle cell types (i.e., cardiac, skeletal, and smooth muscle cells) (Table 1) (Scherer et al., 1994, 1996; Tang et al., 1996). Certain cell types (namely smooth muscle) have expression of all three proteins possibly due to the hybrid fibroblastic/muscle-like nature of this cell. The expression profiles of these proteins in mammalian tissues is intriguing in light of the following observations: 1) despite the divergence of the Cav-2 sequence, it is selectively detected in Cav-1 expressing tissues; and 2) the highly non-overlapping expression patterns of Cav-1 and -3 indicates that the two proteins might have disparate functions in vivo in spite of their high degree of identity.

[1] Abbreviations: VIP-21, vesicular integral protein of 21 kDa; Cav, caveolin; ER, endoplasmic reticulum; N-MAD, N-terminal membrane attachment domain; C-MAD, C-terminal membrane attachment domain; GPI, glycosylphosphatidylinositol; SV40, simian virus 40; VAMP, vesicle-associated membrane protein; kb, kilobase; SRE, sterol regulatory element; LDL, low-density lipoprotein; HDL, high-density lipoprotein; SR-BI, class B type I scavenger receptor; eNOS, endothelial nitric-oxide synthase; PKC, protein kinase C; EGF-R, epidermal growth factor receptor; CSD, caveolin scaffolding domain; CBD, caveolin binding domain; SH, Src homology; NO, nitric oxide; Ach, acetylcholine; GPCR, G-protein-coupled receptor; MAP, mitogen-activated protein; LOH, loss of heterozygosity; LGMD-1C, limb-girdle muscular dystrophy 1C; DG, dystrophin-glycoprotein; EM, electron microscopic; MEF, mouse embryonic fibroblast; KO, knockout; RMD, rippling muscle disease; L-NAME, N^{ω}-nitro-L-arginine methyl ester; MORF, modifier of raft function; AKAP, A-kinase anchor protein; SNAP, soluble NSF (*N*-ethylmaleimide-sensitive factor) attachment protein; SNARE, soluble NSF (*N*-ethylmaleimide-sensitive factor) attachment protein receptor.

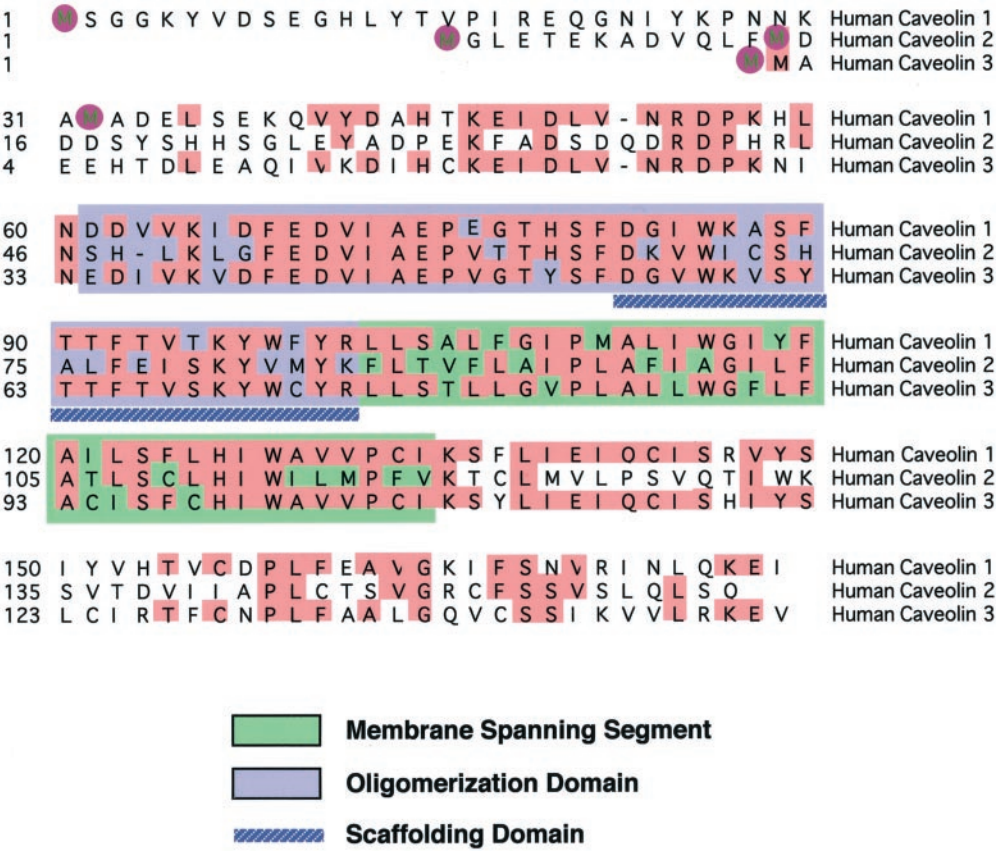

FIG 3. Sequence alignment of the human caveolin gene family. An alignment of the protein sequences of human caveolin-1, -2, and -3 is shown. Identical residues are boxed and highlighted in red. Note that caveolin-1 and -3 are most closely related, while caveolin-2 is divergent. Translation initiation sites are circled. In addition, the positions of the membrane-spanning segment (green), the oligomerization domain (blue), and the scaffolding domain (a subregion of the oligomerization domain—hashed blue) are indicated.

C. Structural Properties of the Caveolins

In several ways, the caveolins are highly unusual proteins, whose overall structure and membrane topology has thus far remained recalcitrant to a complete molecular analysis. Based on a compilation of work conducted mainly on Cav-1 as the archetypal caveolin, two main insights have emerged: 1) Cav-1 is a nonconventional membrane-spanning protein; and 2) it exists primarily as a higher ordered oligomeric complex of ~14 to 16 monomers.

1. Caveolin Membrane Topology. Early work on caveolin-1 showed that it is resistant to extraction with sodium carbonate, a property indicative of its integral association with the plasma membrane (Kurzchalia et al., 1992; Sargiacomo et al., 1993). Based on various topological analyses, it is now well accepted that the N and C termini of Cav-1 remain cytoplasmic. For example, *i*) antibodies recognizing the N and C termini of Cav-1 can bind the protein only when cells are permeabilized with detergents (Dupree et al., 1993); *ii*) membrane-associated Cav-1 remains sensitive to proteolysis, a condition indicative of cytoplasmically directed N and C termini (Monier et al., 1995); and *iii*) there are known phosphorylation and palmitoylation sites in the N-and C-terminal domains of Cav-1, respectively (Dietzen et al., 1995; Li et al., 1996b). By extension, it is reasonable to assume that Cav-1 is a double-pass membrane-spanning protein. However, cell-surface biotinylation of cells shows no labeling of caveolin-1, indicating the inaccessibility of its domains to the extracellular milieu (Sargiacomo et al., 1995). Analysis of the Cav-1 protein sequence indicates that it contains a single hydrophobic region (residues 102–134). As a 32-amino acid stretch is not long enough to enable a complete hairpin loop through the lipid bilayer, a topological view of Cav-1 that took into account its tight association with the plasma membrane was a conundrum.

In a series of biochemical experiments, Kurzchalia and colleagues shed some light on this issue by finding that Cav-1 is inserted into membranes cotranslationally via the classical endoplasmic reticulum (ER) translocation apparatus (Monier et al., 1995). This process is dependent on the 32-residue hydrophobic domain of Cav-1, which acts as a "signal peptide" (note: Cav-1 does not contain a classical N-terminal signal sequence). Interestingly, the N terminus of the protein is required for its hairpin loop configuration, as fusion of the N-terminal domain from a secreted protein (growth hormone) causes Cav-1 to take on a membrane-spanning topology (Monier et al., 1995).

TABLE 1
Overview of mammalian caveolin protein products

Mammalian Protein	Length (AA)	% Similar (identical) to Human Cav-1	% Similar (identical) to Human Cav-2	Expression Patterns	References
Cav-1	178	100 (100)	40 (59)	Ubiquitous; Cav-1 and -2 are co-expressed and are highest in adipocytes, endothelia, smooth muscle cells, and type I pneumocytes	Scherer et al., 1996, 1997
Cav-2	162	58 (38)	100 (100)		
Cav-3	159	85 (65)	39 (60)	Muscle-specific; primarily expressed in Skeletal and cardiac myocytes	Parton 1996; Song et al. 1996b; Tang et al. 1996

Approximate similarity and identity are expressed relative to the human caveolin-1 and -2 proteins.
AA, amino acids. Modified from Razani et al., 2000b.

A holistic view that incorporates all of these observations led investigators to propose an incomplete hairpin structure for Cav-1, where the 32-amino acid hydrophobic domain acted as the putative "membrane-spanning domain" of the protein. Through exhaustive mutational/deletional analyses, recent evidence has elaborated on and complicated this initial model. Fusion proteins containing only the N- or C-terminal domains of Cav-1 (i.e., lacking the transmembrane domain) are also able to bind tightly to membranes (Luetterforst et al., 1999; Schlegel et al., 1999a; Schlegel and Lisanti, 2000). Further deletion analysis shows that residues 82–101 of the N terminus and residues 135–150 of the C terminus are the minimal regions mediating membrane attachment (Schlegel and Lisanti, 2000). These regions are now known as the N-terminal membrane attachment domain (N-MAD) and the C-terminal membrane attachment domain (C-MAD). C-MAD is a membrane attachment domain that directs the *trans*-Golgi localization of Cav-1, whereas N-MAD specifically directs Cav-1 to caveolae membranes. In addition, Cav-1 is palmitoylated on three cysteine residues on the C terminus (133, 143, and 156) (Dietzen et al., 1995). Although these lipid modifications are not necessary for the membrane binding or caveolar localization of Cav-1 (Schlegel and Lisanti, 2000), they act to stabilize the overall structure of the protein at the membrane (see below) (Monier et al., 1996). The present view of caveolin-1's structure at the plasma membrane is shown in Fig. 4.

2. Caveolin Oligomerization. Using velocity gradient centrifugation, it was first discovered that caveolin-1 migrates as a high molecular weight complex of approximately 350 to 400 kDa in vivo (Monier et al., 1995; Sargiacomo et al., 1995). This complex was exclusively composed of the Cav-1 protein and was dissociated only under harsh detergent treatment at elevated temperatures, indicating the presence of a highly stable caveolin-1 homo-oligomer of approximately 14 to 16 monomers (Monier et al., 1995; Sargiacomo et al., 1995; Li et al., 1996c). Furthermore, pulse-chase analysis showed that the complex was formed relatively rapidly after synthesis of Cav-1 in the ER and prior to completion of Golgi transit (Monier et al., 1995).

In vitro and in vivo reconstitution experiments of various deletions of the Cav-1 molecule localized the region responsible for oligomerization to residues 61–101, which was appropriately named the "oligomerization domain" (Sargiacomo et al., 1995; Song et al., 1997b) (Fig. 4).

3. Structural Relationships between the Caveolins. Although it is well established that Cav-1 can form large homo-oligomeric complexes in vivo, Cav-1 has recently been shown to undergo higher ordered interactions with caveolin-2 as well. Cav-1 and -2 can interact to form very stable high molecular mass hetero-oligomers, akin to the

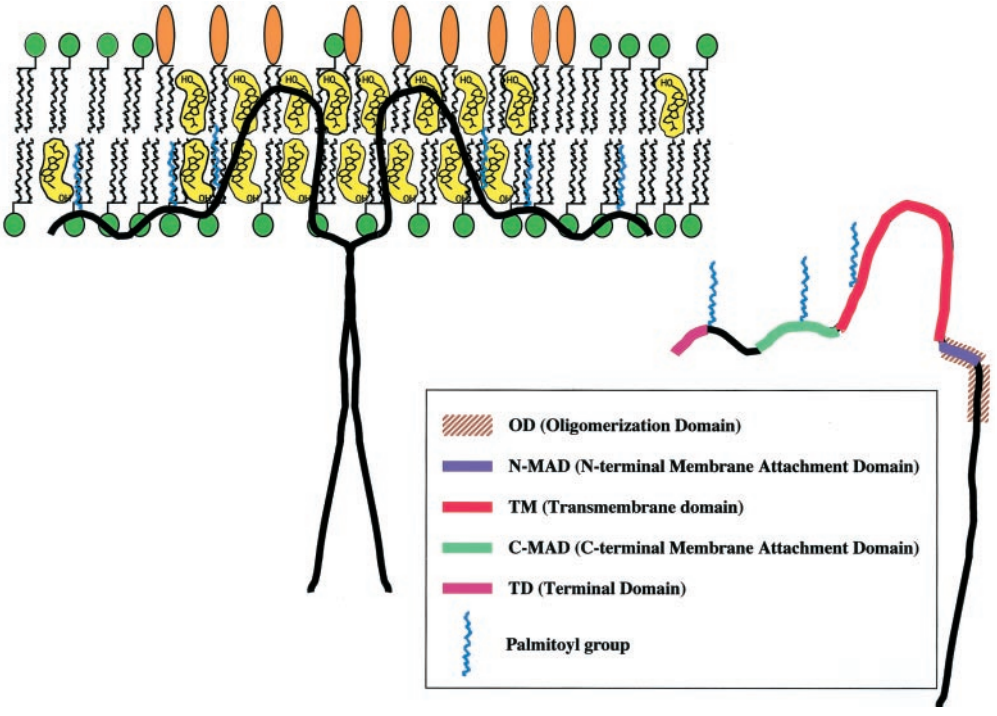

FIG 4. The current view of caveolin-1 membrane topology. Caveolin-1 exists as either a homo-oligomer of ~14 to 16 monomers (shown as a dimer for simplicity) or as a hetero-oligomer with caveolin-2 (not shown). Via its hydrophobic trans-membrane (TM) domain (red), Cav-1 is believed to penetrate the membrane. The protein is also bound to membranes through tight association between the N-MAD and C-MAD (shown in lavender and green, respectively). Homo-oligomerization is mediated by a 40-amino acid stretch (residues 61–101; which incidentally encompasses N-MAD) known as the oligomerization domain (OD) (hashed brown). Adjacent oligomers interact via the terminal domain (TD) (purple). Sites of palmitoylation (Cys133, -143, and -156) are shown in blue.

large homo-oligomeric complexes observed for Cav-1 (Scherer et al., 1997). Interestingly, in the absence of Cav-1, Cav-2 is not capable of forming large homo-oligomers and rather exists in a monomeric/dimeric form and is retained at the level of the Golgi compartment (Scherer et al., 1996; Mora et al., 1999; Parolini et al., 1999). Upon Cav-1 expression, the Cav-1 and Cav-2 proteins form a large complex and are found predominantly localized to plasma membrane caveolae (Scherer et al., 1996; Mora et al., 1999; Parolini et al., 1999). Therefore, Cav-2 is dependent on caveolin-1 both structurally and for appropriate subcellular trafficking (Mora et al., 1999; Parolini et al., 1999). Based on the primary sequence, the oligomerization domain of Cav-2 is ~50% identical/75% similar to that of Cav-1, and it was believed that oligomer formation between the two proteins is mediated by their respective oligomerization domains. However, recent deletion mapping studies surprisingly indicate that the interaction is mediated by the membrane-spanning domains of Cav-1 and -2 (Das et al., 1999). The tissue-specific expression of Cav-1 and Cav-2 is highly coregulated (i.e., all tissues with Cav-1 expression are also the primary sites of Cav-2 expression) (Scherer et al., 1996). Therefore, despite the well established fact that Cav-1 can form large homo-oligomeric complexes in vivo, it seems more likely that the Cav-1 protein is constitutively associated with Cav-2 under physiological conditions.

Based on its high sequence similarity to Cav-1 (especially in the oligomerization and membrane-spanning domains) (see Fig. 3), caveolin-3 has been thought to have Cav-1-like structural properties. Indeed, when overexpressed, Cav-3 can oligomerize to form a ~350- to 400-kDa complex and is targeted to caveolae (Tang et al., 1996). However, the two proteins are not simply redundant in all respects, e.g., the C-terminal domain of Cav-1 can bind to full-length Cav-1 oligomers (homotypic interactions) but not full-length Cav-3 oligomers (Song et al., 1997b), and unlike Cav-1, Cav-3 does not form a complex with Cav-2 (Das et al., 1999).

Therefore, despite their high identity, it appears that Cav-1 and -3 have distinct structural properties that might affect their localization and function in the cell. For example, in the widely used myocyte cell line, C2C12, Cav-1 and -3 are both expressed and colocalized to caveolae in the myoblast stage. Upon myocyte differentiation, however, Cav-1 levels steadily decline (albeit remaining associated with the cell periphery) while Cav-3 levels increase and associate with the developing T-tubule network (Parton et al., 1997).

IV. Caveolar Biogenesis

A. The Role of Cholesterol

The crucial role of cholesterol in caveolar biogenesis has been evident for some time. Treatment of cells with cholesterol binding agents such as nystatin, filipin, or cyclodextrin leads to a complete ablation of morphologically identifiable caveolae (Rothberg et al., 1992;

Schnitzer et al., 1994; Hailstones et al., 1998). Furthermore, absolute cellular levels of cholesterol need to rise above a certain threshold level before caveolae formation can occur (Hailstones et al., 1998). Although caveolae microdomains are highly enriched in cholesterol, the selective requirement for cholesterol in caveolar biogenesis is not straightforward. Recent reports have clearly shown that manipulation of cellular cholesterol levels also has effects on the biogenesis of clathrin-coated endocytic vesicles and synaptic vesicles, membrane entities that differ in many respects from lipid rafts and caveolae (Rodal et al., 1999; Thiele et al., 2000). If the formation of many types of post-Golgi-derived vesicles depends on critical levels of cholesterol, the formation of caveolae must involve more than just the role played by cholesterol.

B. The Role of the Caveolins

Overexpression of caveolin-1 in cells lacking endogenous caveolin/caveolae (lymphocytes and transformed fibroblasts) results in the de novo production of ~50- to 100-nm membrane invaginations and vesicles (Fra et al., 1995b; Engelman et al., 1997; Li et al., 1998). Conversely, the targeted down-regulated caveolin-1 in cells with endogenous caveolae results in the loss of caveolae (Galbiati et al., 1998; Liu et al., 2001). These experiments clearly link caveolin expression with caveolae formation.

Several characteristics of caveolin-1 suggest the way in which it transforms the morphology of the plasma membrane into flask-shaped caveolae. Cav-1 binds the two primary components of lipid rafts, namely cholesterol and sphingolipids, both in vitro and in vivo (Fra et al., 1995a; Murata et al., 1995; Thiele et al., 2000). In fact, cholesterol has an unusually high affinity toward Cav-1, remaining associated with caveolin oligomers even in the presence of harsh detergents such as SDS (Murata et al., 1995). A high local concentration of cholesterol and caveolin maintained at a critical level could provide the appropriate lipid-protein microenvironment for membrane invagination (Fig. 5).

The tendency of caveolins to form high molecular weight oligomeric complexes likely contributes to caveolae formation. As mentioned before, caveolin-1 is able to form extremely strong detergent-resistant homo-oligomeric complexes composed of ~14 to 16 monomers (Monier et al., 1995; Sargiacomo et al., 1995). If these oligomers are purified from plasma membrane lysates and detergents are subsequently removed by dialysis, the oligomers self-associate into very large/sedimentable complexes of ~20- to 40-nm in diameter (Sargiacomo et al., 1995). Furthermore, deletion analysis has revealed that the C-terminal domain of Cav-1 (namely residues 135–178), is able to interact with the full-length Cav-1 molecule (Song et al., 1997b; Schlegel and Lisanti, 2000). Taken together, this evidence suggests that caveolin oligomers can form higher order oligomer-oligomer complexes by interactions of the respective C-terminal domains. This meshwork of caveolin protein complexes most likely elicits the bending of the plasma membrane, a process similar to that of the clathrin triskelion in coated-pit formation (Fig. 5).

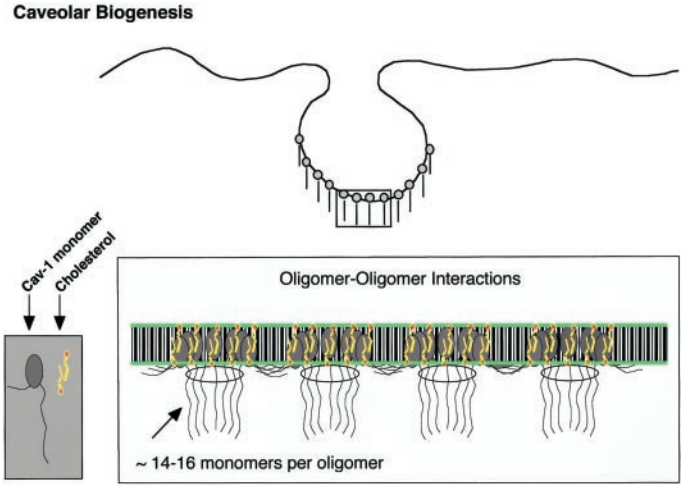

FIG 5. Caveolin-1 oligomerization and caveolae biogenesis. At the level of the ER, caveolin-1 self-associates to form high molecular mass homo-oligomers that contain ~14 to 16 individual caveolin-1 molecules. These caveolin-1 oligomers represent the functional assembly units of caveolae. Then, these caveolin-1 homo-oligomers undergo a second stage of oligomerization during transport to the plasma membrane, most likely at the level of the *trans*-Golgi. In this second stage of oligomerization, individual caveolin-1 oligomers interact with each other via their C-terminal domains, forming an extensive network or meshwork on the underside of the plasma membrane. This large meshwork of oligomers may act synergistically with cholesterol (yellow) to distort the membrane and to drive the invagination of caveolae.

V. Functional Significance of Caveolae/Caveolins

Since their discovery in the 1950s, the function of caveolae has been a matter of speculation. Consequently, numerous roles have been attributed to these microdomains and their caveolar marker protein, the caveolins. Here, we will discuss the most thoroughly studied roles of caveolae and their potential relevance to physiology.

A. Vesicular Transport

1. Transcytosis. Based purely on the vesicular morphology and abundance of caveolae in endothelial cells (Palade 1953), caveolae were hypothesized to function as conduits for the general transport of proteins through capillary cells (i.e., transcytosis) (Fig. 6) (Simionescu et al., 1975). The primary approach in these initial reports and numerous follow-up studies involves the use of labeled "tracer" serum proteins (e.g., gold-conjugated albumin) and following their dynamic interaction with the capillary endothelium via transmission electron microscopy (Simionescu et al., 1975; Ghitescu and Bendayan, 1992; Schnitzer et al., 1994; Predescu et al., 1997, 1998). Despite very convincing time-lapse imaging of caveolae-mediated tracer transport to the abluminal side of endothelial cells, the functionality of caveolae as transcy-

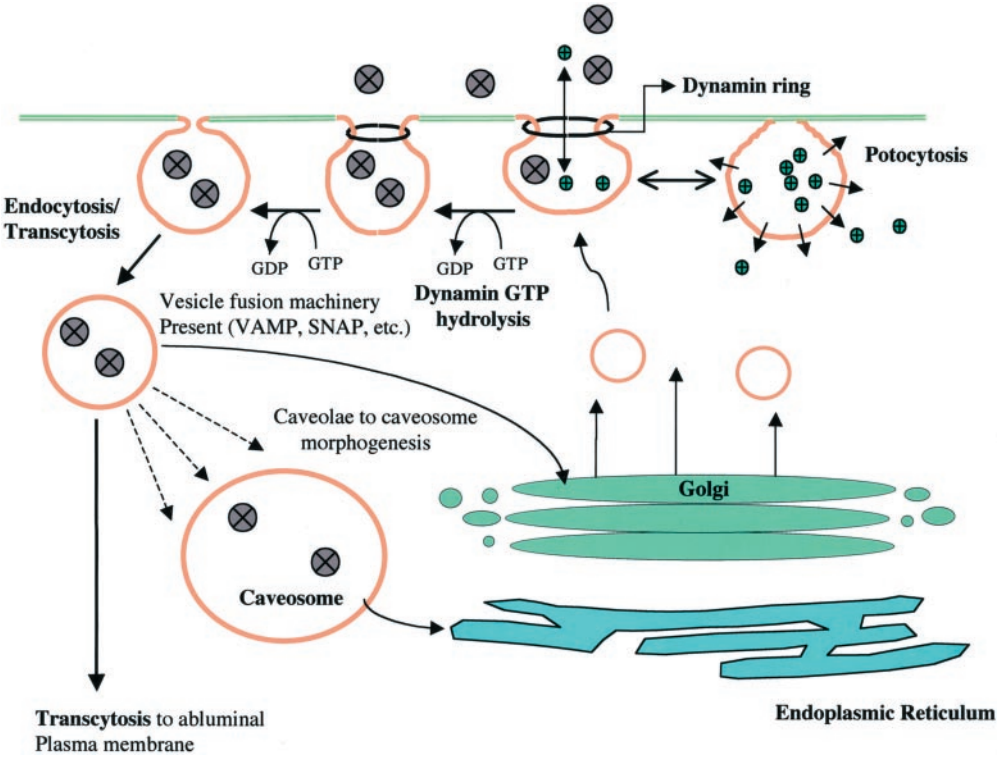

FIG 6. Caveoale vesicular trafficking: transcytosis, endocytosis, and potocytosis. Caveolae appear to mediate the selective uptake and transport of several molecules via different processes (transcytosis, endocytosis, and potocytosis). In transcytosis, caveolae transport proteins from the luminal side of the endothelial cell to the interstitial compartment for subsequent uptake by underlying tissues. In caveolae-mediated endocytosis (distinct from that of clathrin-coated pits), caveolae bud off from the plasma membrane and fuse with various intracellular compartments. Possible transport routes include the recently characterized caveolae-caveosome-ER pathway. In potocytosis, caveolae mediate the uptake of small solutes (<1 kDa) by pinching off but remaining associated with the plasma membrane. The molecular machinery involved in this caveolar fission/fusion is the same as that used for numerous other vesicular transport processes with requirements for dynamin, VAMP, SNAP-25, the SNARE complex, and GTP hydrolysis.

totic vesicles has been contentious. It is not clear whether this observed mode of transport is specific to caveolae or is simply a nonspecific component of bulk fluid flow, which would include paracellular transport. Furthermore, it has been argued that the observed tracer labeling of invaginated caveolae (i.e., cytoplasmic caveolae in transit to the abluminal side) could actually be labeling of a continuation of the plasma membrane only appearing to be intracellular due to microscopic sectioning artifacts.

To more directly address these issues, Schnitzer and colleagues (2001) have developed antibodies capable of specifically labeling the extracellular (luminal) domains of proteins residing in endothelial cell caveolae. In situ labeling experiments showed that these antibodies are capable of completely and rapidly crossing the capillary wall directly through caveolae; bulk fluid flow (as determined by nonspecific antibodies) occurred significantly more slowly (Schnitzer, 2001). These observations show that caveolae-mediated transport is a major and kinetically preferred route for the transcytosis of certain proteins.

2. Endocytosis. Caveolae have also been implicated in endocytic processes. Traditionally, endocytosis has been almost entirely associated with clathrin-coated vesicle transport and the molecular mechanisms underlying this process have been studied in detail (for review, see Takei and Haucke, 2001). Numerous receptors and their cognate ligands are taken up via this pathway leading to termination or desensitization of signaling cascades, among other things. However, it has become clear that certain receptors and extracellular macromolecules are exclusively transported via caveolae rather than clathrin-coated pits (Fig. 6). The first demonstration of this selectivity came when investigators found that caveolae can bind and internalize cholera and tetanus toxins (Montesano et al., 1982). Biochemical evidence bolstered this argument when it was shown that treatment of cells with cholesterol binding agents (nystatin or filipin) abrogated the endocytosis of certain macromolecules (e.g., albumin) without affecting the uptake of clathrin-dependent ones (Schnitzer et al., 1994). Similar results have been seen with several membrane-bound proteins including alkaline phosphatase (Montesano et al., 1982; Anderson et al., 1992; Parton et al., 1994). This selectivity is in large part due to the caveolar localization of the cognate receptor for these molecules [e.g., the glycosphingolipid GM1 in the case of cholera toxin or the glycosylphosphatidylinositol (GPI) modification of alkaline phosphatase] (Fig. 6) (see also Table 2 for a more complete list of caveolae-localized proteins).

It appears that a variety of organisms have evolved strategies to take advantage of the caveolae endocytic machinery: simian virus 40 (SV40) and certain strains of *Escherichia coli* have also been shown to be endocytosed by receptors resident in caveolae (Anderson et al., 1996; Stang et al., 1997; Shin et al., 2000). The relatively small size of caveolae (~50–100 nm) obviously places limitations on the endocytosis of such large entities. In the case of bacteria, it appears that phagocytic cells circumvent such problems by harboring mechanisms that enable several caveolae to coalesce into significantly larger endocytic microdomains (Shin et al., 2000).

Exactly where in the cell the endocytosed caveolar vesicle targets is not clear. Among the possibilities are direct transport to endosomes with or without further trafficking to other intracellular compartments. For example, in the case of cholera toxin, it is believed that caveolar endocytosis on the apical membrane is followed by retrograde transport to endosomes, the Golgi apparatus, the endoplasmic reticulum, and finally resorting to the basolateral membrane (Lencer et al., 1999). However, this trafficking scheme is not true for all caveolae-mediated endocytosis. It has been shown that the budded caveolae containing SV40 eventually merge with larger nonendosomal nonlysosomal caveolin-1 containing organelles that target to the endoplasmic reticulum (Pelkmans et al., 2001). These organelles were termed "caveosomes" and are the first indication of distinct compartments formed from internalized caveolae (Fig. 6). This is consistent with earlier data that under certain conditions caveolins can either directly traffic from the plasma membrane to ER/Golgi compartments or localize to nonendosomal vesicles (Conrad et al., 1996; Roy et al., 1999).

3. Mechanisms of Endocytosis/Transcytosis. A variety of proteins have been identified that comprise the vesicular fission/fusion machinery required for endocytosis and transcytosis. In a series of studies primarily by Schnitzer and colleagues, it was shown that caveolae harbor the same components used for the budding and docking of other vesicles (Schnitzer et al., 1995b). Early indications for the presence of such classical transport machinery was based on the observation that *N*-ethylmaleimide (NEM) can inhibit the transcytosis/endocytosis of endothelial cell caveolae (Predescu et al., 1994; Schnitzer et al., 1995a). Purification of these caveolar microdomains showed that several components used by cells in general vesicle formation, docking, and fusion (e.g., NSF, SNAP, VAMP, and GTPases) are concentrated in caveolae and possibly associated with caveolin-1 (Schnitzer et al., 1995b; Predescu et al., 2001) (see also Table 2). Furthermore, cleavage of VAMP by specific neurotoxins abrogates the endocytosis and fusion of caveolae with intracellular compartments (McIntosh and Schnitzer, 1999). Therefore, it has become clear that caveolae can co-opt the same mechanisms used in the trafficking of other vesicles to transport cargo throughout the cell, e.g., from the plasma membrane to internal compartments (Fig. 6).

More recent work has shed light on the processes leading to caveolar fission. Purified endothelial cell plasma membranes incubated with cytosol and GTP lead to the budding/fission of caveolae (Schnitzer et al., 1996). The GTP-dependent factor in the cytosol was shown to be dynamin, previously implicated in the endocytosis of clathrin-coated pits (Henley et al., 1998; Oh et al., 1998). Microinjection of anti-dynamin antibodies into cells prevented the internalization of clathrin-coated pits, as well as caveolae (Henley et al., 1998). In vivo, it appears that dynamin localizes to the conspicuous annular necks of membrane-bound caveolae seen by electron microscopy (Henley et al., 1998; Oh et al., 1998). Given the role of dynamin in vesicular budding, its position at the caveolar "necks" is not surprising (Fig. 6).

Despite the involvement of the classical fission/fusion apparatus in caveolar endocytosis, the steps leading to invagination and vesicle formation remain fragmented. As mentioned before, caveolin-1 along with cholesterol most likely provides the structural force for the invagination of caveolae. However, the regulatory mechanisms mediating the transition from membrane-bound caveolae to free intracellular vesicles are at present unknown. How do caveolae sense the need to undergo endocytosis or remain statically attached to the plasma membrane, and what are the stimuli that trigger caveolar endocytosis? Future work will be needed to provide the links between the caveolins, dynamins, and such molecular sensors.

4. Potocytosis. Caveolae have also been implicated in a unique form of solute uptake, termed potocytosis (or "cellular drinking"). First championed by Anderson and colleagues (1992), potocytosis is the process by which cells can transport small molecules (<1 kDa) without having to endocytose vesicles to internal endosomal/lysosomal compartments. Using microscopy, they demonstrated that the folate receptor, a GPI-linked protein, primarily localizes to caveolae (Rothberg et al., 1990). Remarkably, caveolae then seem to facilitate the uptake of folate by closing their necks, albeit remaining associated with the plasma membrane (Anderson et al., 1992). In this way, a highly concentrated pool of folate is created, which is subsequently fluxed to the cytoplasm by a three-step process: 1) lowering of caveolar pH by a caveolae-localized proton pump, 2) low pH dissociation of folate from its receptor, and 3) flux into the cytoplasm by a caveolae-localized anion carrier (Kamen et al., 1991) (Fig. 6). At present, the mechanism of the dynamic closing and opening of the caveolar "mouth" is not understood. Furthermore, there is controversy as to whether cells even use potocytosis in the uptake of small molecules or simply co-opt classical receptor-mediated endocytic processes (Mayor et al., 1998). More detailed analysis of the general applicability of caveolae to the uptake of other small molecules needs to be conducted before

TABLE 2
List of caveolae-localized and caveolin-interacting molecules

Caveolar Localization	M	B	Interact with Caveolins	References
G-protein-coupled receptors:				
β_1 and β_2 adrenergic receptor	+	+	+	Schwencke et al., 1999; Ostrom et al., 2000b; Rybin et al., 2000
Bradykinin B2 receptor	+	+		Haasemann et al., 1998; Ju et al., 2000
Endothelin type A	+	+	+	Chun et al., 1994; Okamoto et al., 2000
Cholecystokinin	+			Roettger et al., 1995
m-2 acetylcholine		+		Rybin et al., 2000
Ca^{2+}-sensing receptor	+			Kifor et al., 1998
Adenosine A(1) receptor		+		Lasley et al., 2000
G-proteins				
$G\alpha_s$		+	+	Donati et al., 2001; Li et al., 1996a; Lisanti et al., 1994b; Rybin et al., 2000
$G\alpha_{i1}$		+	+	Lisanti et al., 1994b; Li et al., 1996a; Song et al., 1997a; Rybin et al., 2000
$G\alpha_{i2}$		+	+	Lisanti et al., 1994b; Li et al., 1996a; Rybin et al., 2000
G_o			+	Li et al., 1996a
$G\beta\gamma$		+		Lisanti et al., 1994b; Schwencke et al., 1999
Gq		+		Lisanti et al., 1994b; Pike and Miller, 1998; Oh and Schnitzer, 2001
Membrane proteins				
Insulin receptor		+	+	Yamamoto et al., 1998; Gustavsson et al., 1999; Nystrom et al., 1999
PDGF receptor	+	+	+	Liu et al., 1996b; Yamamoto et al., 1999
EGF receptor		+		Mineo et al., 1996, 1999
VEGF receptor	+	+		Feng et al., 1999
p75 NGF receptor		+	+	Bilderback et al., 1997; Huang et al., 1999
Trk A		+		Huang et al., 1999
c-Neu			+	Engelman et al., 1998b
TGFβ type I and II receptors	+	+	+	Razani et al., 2001b
RAGE	+	+		Schmidt et al., 1992; Lisanti et al., 1994b; Stitt et al., 2000
GLUT-4	+	+		Scherer et al., 1994; Gustavsson et al., 1996; Ros-Baro et al., 2001
Ca^{+2}-ATPase	+			Tachibana and Nawa, 1992; Fujimoto, 1993; Schnitzer et al., 1995c
H^+-ATPase	+			Mineo and Anderson, 1996
TFPI (Tissue Factor Pathway inhibitor)	+	+		Lupu et al., 1997; Crawley et al., 2000
Flotillin-1 and -2		+	+	Bickel et al., 1997; Volonte et al., 1999
AMR-R (Autocrine motility factor receptor)	+			Benlimame et al., 1998
PV-1	+			Stan et al., 1999
P-glycoprotein		+	+	Demeule et al., 2000
L-type Ca^{2+} channel	+	+		Darby et al., 2000; Daniel et al., 2001
Plasma membrane Ca^{2+} pump	+	+		Darby et al., 2000
Kv1.5 voltage-gated K^+ channel	+	+		Martens et al., 2001
Membrane-type 1 matrix metalloproteinase (MT1-MMP)		+		Annabi et al., 2001
Apolipoprotein E receptor 2 (apoER2)		+		Riddell et al., 2001
CD48 (GPI)	+	+		Shin et al., 2000
Nonreceptor tyrosine kinases				
Src		+	+	Sargiacomo et al., 1993; Li et al., 1996a
Fyn	+	+	+	Sargiacomo et al., 1993; Shenoy-Scaria et al., 1994; Li et al., 1996a
Yes		+		Sargiacomo et al., 1993
Lck		+		Lisanti et al., 1994b; Shenoy-Scaria et al., 1994
Hck		+		Robbins et al., 1995a; b
Lyn		+		Lisanti et al., 1994b
JAK2		+		Lisanti et al., 1994b
Tyk2		+		Ju et al., 2000
STAT3		+		Ju et al., 2000
Nonreceptor Ser/Thr kinases				
PKA	+	+	+	Razani et al., 1999
Raf		+		Mineo et al., 1996
MEK			+	Engelman et al., 1998a
ERK			+	Engelman et al., 1998a
PI 3-kinase		+	+	Lisanti et al., 1994b; Zundel et al., 2000
GRK		+	+	Carman et al., 1999
PKCα, δ, ε		+		Lisanti et al., 1994b; Kifor et al., 1998; Rybin et al., 1999

TABLE 2
Continued

Caveolar Localization	M	B	Interact with Caveolins	References
Other enzymes				
Adenylyl cyclase		+	+	Fagan et al., 2000; Ostrom et al., 2000b; Rybin et al., 2000
eNOS	+	+	+	Garcia-Cardena et al., 1996b, 1997; Liu et al., 1996a; Shaul et al., 1996
nNOS	+		+	Venema et al., 1997; Daniel et al., 2001
Phopholipase D2		+	+	Czarny et al., 1999
Phospholipase C-γ1	+	+		Jang et al., 2001
Prostacyclin synthase (PGI2)	+	+	+	Spisni et al., 2001
Cyclooxygenase-2 (COX-2)	+	+	+	Liou et al., 2001
GTPases				
H-Ras	+	+	+	Mineo et al., 1996; Song et al., 1996a; Prior et al., 2001
Rap1		+		Chang et al., 1994; Lisanti et al., 1994b
Rap2		+		Lisanti et al., 1994b
RhoA		+	+	Gingras et al., 1998
Cdc42		+		Gingras et al., 1998
Cellular proteins/adaptors				
PIP, PIP2	+	+		Pike and Casey, 1996; Pike and Miller, 1998; Waugh et al., 1998
Shc		+	+	Lisanti et al., 1994b; Smith et al., 1998; Wary et al., 1998
SOS		+		Smith et al., 1998
GRB2		+		Lisanti et al., 1994b; Smith et al., 1998
IRS-1				Smith et al., 1998
E-cadherin	+	+	+	Galbiati et al., 2000a
β-catenin	+	+	+	Galbiati et al., 2000a
γ-catenin	+	+	+	Galbiati et al., 2000a
Calsequestrin	+	+	+	Darby et al., 2000; Daniel et al., 2001
Calreticulin	+	+	+	Darby et al., 2000; Daniel et al., 2001
Nuclear proteins				
Estrogen receptor		+	+	Schlegel et al., 1999b; Chambliss et al., 2000
Androgen receptor		+	+	Lu et al., 2001
Structural proteins				
Actin	+	+		Chang et al., 1994; Lisanti et al., 1994b
Annexin II and IV		+		Sargiacomo et al., 1993; Lisanti et al., 1994b; Schnitzer et al., 1995b
VAMP-2		+		Schnitzer et al., 1995b; McIntosh and Schnitzer, 1999
SNARE	+	+		Schnitzer et al., 1995b; Predescu et al., 1997
SNAP		+		Schnitzer et al., 1995b
NSF	+	+		Schnitzer et al., 1995b; Predescu et al., 1997
Dynamin	+	+		Predescu et al., 1997; Henley et al., 1998; Oh et al., 1998
MAL		+		Millan et al., 1997a,b
Other proteins				
Atrial Natriuretic Peptide	+			Page et al., 1994

M, morphological evidence; B, biochemical evidence.

potocytosis can be considered a process distinct from caveolar endocytosis.

B. Cellular Cholesterol Homeostasis

As briefly described above, a unique relationship exists between caveolins/caveolae and cholesterol. Ever since the discovery of caveolin-1 as a marker of caveolae, it has been known that caveolae are highly sensitive to cholesterol depletion; treatment of cells with cholesterol binding agents flattens these structures and disaggregates their caveolin-rich "striated coats" (Rothberg et al., 1992). The first indication of a direct link between the caveolins and cholesterol was demonstrated in vitro, where purified caveolin-1 was shown to reconstitute only into lipid vesicles containing cholesterol (Murata et al., 1995; Li et al., 1996c). Further analysis found that Cav-1 can actually form a complex with cholesterol in these lipid mixtures, on the order of ~1 to 2 cholesterol molecules per caveolin molecule (Murata et al., 1995). Recently, a photo-activatable form of cholesterol was shown to cross-link a ~21- to 24-kDa band in cells (identified as Cav-1), thereby making caveolin-1 one of only a few proteins with a demonstrated ability to bind cholesterol in vivo (Thiele et al., 2000). Not surprisingly, the intricate relationship with cholesterol renders Cav-1 highly sensitive to cholesterol perturbations, both in terms of its subcellular localization and transcriptional regulation.

1. The Effect of Cholesterol on Caveolin-1. Treatment of cells with cholesterol oxidase, which selectively converts caveolar cholesterol to cholestenone, causes the movement of caveolin-1 to the ER and subsequently to the Golgi compartment; removal of cholesterol oxidase allows the return of Cav-1 and free cholesterol to caveolae (Smart et al., 1994; Conrad et al., 1996). Although it is not known how Cav-1 can retreat intracellularly, it is clear that its subcellular transport is intricately linked to cholesterol transport.

Recent evidence suggests that caveolin-1 expression is also dependent on cellular cholesterol content. Depletion of cellular cholesterol by simvastatin or cyclodextrin causes a reduction in the number of caveolae and a concomitant drop in caveolin-1 protein and mRNA levels (Hailstones et al., 1998). Rather than depleting cellular cholesterol, Fielding and Fielding used cholesterol-loading to dissect this regulation even further (Bist et al., 1997; Fielding et al., 1997, 1999). Incubation of fibroblasts with low density lipoproteins, a way of loading cells with free cholesterol, led to an increase in Cav-1 mRNA levels (Fielding et al., 1997). Mutational/deletion analysis of the 1-kb upstream caveolin promoter revealed that two putative sterol regulatory elements (SREs) are necessary for robust transcriptional activation by free cholesterol; electrophoretic mobility shift assays further defined one particular SRE that is bound by the transcription factor SRE-binding protein 1 (SREBP-1), a major mediator of cholesterol-dependent transcriptional responses (Bist et al., 1997). Taken together, the above results suggest a pivotal role for caveolins/caveolae in the transport and regulation of cellular cholesterol levels.

2. Intracellular Transport of de Novo Synthesized cholesterol. De novo synthesis of cholesterol occurs in the ER, and upon membrane partitioning, it is rapidly transported to various membrane compartments, of which the plasma membrane is a major site (containing up to 90% of cellular cholesterol at steady state) (Simons and Ikonen, 2000). However, much less is known about the molecular machinery and trafficking pathways mediating this cholesterol transport. Based on a variety of observations, it is believed that cholesterol is predominantly transported to the cell surface by a Golgi-independent route (DeGrella and Simoni, 1982; Kaplan and Simoni, 1985; Urbani and Simoni, 1990; Heino et al., 2000). Considering its high binding capacity for cholesterol, caveolin-1 is a potential mediator of this function.

Indeed, by using radiolabeled acetate (a precursor in the cholesterol biosynthetic pathway), Smart and colleagues (1996) were able to show that newly synthesized cholesterol is distributed to the bulk plasma membrane only after initial transport to the caveolae membrane system (Fig. 7). Furthermore, this cholesterol transport was mediated by a nonvesicular protein complex, which includes caveolin-1 and several chaperone proteins of the cyclophilin and heat shock protein families (Uittenbogaard et al., 1998) (Fig. 7). Interestingly, the three palmitoylation sites of Cav-1 were necessary for the binding and delivery of cholesterol to caveolae (Uittenbogaard and Smart, 2000). This is an important observation in light of the previous finding that the palmitoylation of Cav-1 is not necessary for its membrane binding or caveolar localization but rather for its stability as an oligomer, a process intricately involved in its cholesterol binding as well (Dietzen et al., 1995; Monier et al., 1996). Based on these results, it is clear that cholesterol trafficking to the cell surface utilizes the

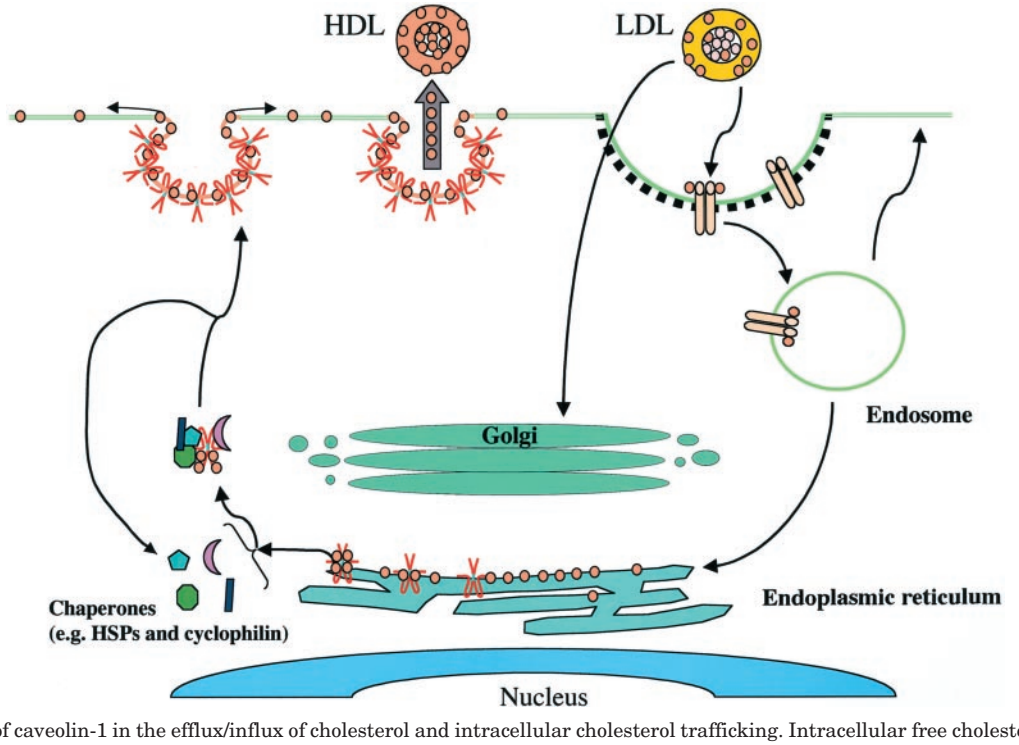

FIG 7. The role of caveolin-1 in the efflux/influx of cholesterol and intracellular cholesterol trafficking. Intracellular free cholesterol is derived from two main sources (externally from LDL by either receptor-mediated endocytosis or by membrane loading) or from de novo synthesis. This cholesterol is then shuttled throughout the cell to all membrane compartments. Caveolin-1 can then bind cholesterol in the ER and transport it to caveolae at the plasma membrane. At this juncture, the cholesterol can either be effluxed to extracellular lipoproteins (namely HDL) or be siphoned into the bulk plasma membrane. Caveolin-1 can then retreat to the ER/Golgi compartments and repeat this transport cycle.

caveolin-1/caveolar system as a "delivery device". It should be noted, however, that many cells with an intact cholesterol transport apparatus (e.g., hepatocytes) have extremely low levels of caveolin-1 (Calvo et al., 2001). Therefore, under physiologic conditions, Cav-1 most likely only acts as a facilitator/expeditor of cholesterol transport necessitating the identification of other pathways in cholesterol trafficking. In line with a facilitatory role, transfection of cells with the caveolin-1 cDNA leads to an ~4- to 5-fold enhancement of caveolar cholesterol levels (Smart et al., 1996).

3. Cholesterol Efflux from Cells. Cellular cholesterol is typically derived from two major sources: de novo synthesis (as described above) or exogenous uptake primarily from serum low-density lipoproteins (LDLs) (Fielding and Fielding, 1997). To maintain steady levels intracellularly, peripheral cells can also efflux excess free cholesterol from the exoplasmic leaflet of the plasma membrane to high-density lipoproteins (HDLs) for delivery to the liver, a process known as reverse cholesterol transport (Fielding and Fielding, 1997). In a series of studies by Fielding and Fielding, it appears that caveolae play an important role in this process as well. If the intracellular cholesterol pool is increased above baseline by incubating cells with cholesterol-donating LDL, excess cholesterol is eventually observed in caveolae; caveolae then act as portals for the efflux of this cholesterol upon incubation of cells with HDL (Fig. 7) (Fielding and Fielding, 1995). Caveolin-1 regulates this process, because antisense-mediated down-regulation of caveolin-1 decreases cellular cholesterol efflux (Fielding et al., 1999; Arakawa et al., 2000). In support of these data, in vivo expression of caveolin-1 in the mouse liver via adenoviral-based strategies causes an increase in plasma HDL cholesterol levels (Frank et al., 2001). Therefore, it appears that Cav-1 enhances the availability and delivery of cholesterol to plasma membrane caveolae for subsequent efflux. At present however, the molecular mechanisms of this efflux process from caveolae to HDL are not known, but most likely involve an association between the HDL particle or its major apolipoprotein, ApoAI, and caveolae.

In this regard, it should be noted that a major component of HDL-mediated cholesterol transfer, SR-BI (the class B type I scavenger receptor) is concentrated in caveolae (Babitt et al., 1997; Graf et al., 1999). SR-BI has been shown to act as an HDL receptor functioning both in the transfer of free cholesterol from the plasma membrane to HDL (Ji et al., 1997; de la Llera-Moya et al., 1999), while also acting as a conduit for the selective uptake of cholesterol esters from HDL into the cell (Acton et al., 1996). Therefore, the role of caveolae in the efflux of free cholesterol could be largely due to the compartmentalization of the SR-BI receptor.

Based on the dual functionality of SR-BI, caveolar localization would also imply that caveolae are important sites of cholesterol ester uptake. Indeed, it has been shown that SR-BI-mediated cholesterol ester uptake predominantly occurs in caveolae (Graf et al., 1999) and that caveolin-1 can regulate this process (Matveev et al., 1999, 2001; Frank et al., 2001). However, many of these results need to be dissected further to understand the physiological role of caveolins/caveolae in tissues that utilize SR-BI for cholesterol influx/efflux processes.

C. Signal Transduction Mechanisms

1. Caveolae As Signalosomes: Compartmentalized Signaling. Caveolin-1 was not only the first protein to be localized to caveolae but due to its apparent involvement in the structural integrity of caveolae was also the first caveolar "marker protein" (Rothberg et al., 1992). The issue that required clarification, however, was whether caveolae could also serve as platforms for the aggregation and/or concentration of other proteins. Clearly, evidence for the presence of other caveolar resident proteins would be important in the understanding of caveolar function. In this regard, Lisanti and coworkers were the first investigators to broadly address this issue (Sargiacomo et al., 1993; Lisanti et al., 1994b). Using the insolubility of caveolae in mild detergents and their buoyancy in sucrose gradients, they were able to biochemically separate caveolae membranes and, in turn, determine the identity of cosegregated proteins. Of the numerous proteins identified in this manner, it was surprising to find that a large majority were signal transduction molecules, some at concentrations manyfold higher than the bulk plasma membrane (Sargiacomo et al., 1993; Lisanti et al., 1994b). This observation led Lisanti and colleagues to put forth the "caveolae/raft signaling hypothesis": the compartmentalization of such molecules has distinct advantages as it provides a mechanism for the regulation of subsequent signaling events and explains *cross-talk* between different signaling pathways (Lisanti et al., 1994a).

In the decade of research since this initial observation, an array of proteins (ranging from receptor tyrosine kinases, G-protein-coupled receptors, ion channels, adaptor proteins, and structural proteins) have now been reported to be preferentially localized to caveolae (see Table 2 for an expanded list of caveolae-localized molecules). It should be noted that although there does not seem to be an absolute criterion for the ability of a protein to localize to caveolae, several of these signaling molecules contain lipid modifications. Well characterized caveolae-associated proteins such as H-Ras, Src family tyrosine kinases, heterotrimeric G-protein α subunits, and endothelial nitric-oxide synthase (eNOS) all harbor one or several myristoyl, palmitoyl, or prenyl groups (Li et al., 1995, 1995a; Feron et al., 1996; Garcia-Cardena et al., 1996b; Song et al., 1996a, 1997a).

As indicated in Table 2, the evidence supporting the caveolar localization of certain proteins is more rigorous (i.e., localization by both biochemical and morphological approaches) than others. As more research is conducted,

this growing list is likely to be revised and appended in many ways. Nevertheless, it is clear that caveolae are involved in the compartmentalization of various signaling pathways and can be considered specialized signaling organelles (or more appropriately, "signalosomes").

2. The Caveolins As Modulators of Signaling. A topic neglected by this signaling concept was the functional significance of the caveolin proteins (were they simply structural bystanders or could they actively contribute in the retention and modulation of signal transduction proteins). After all, the concept of "scaffolding proteins" is not a new one, and the literature is replete with examples of molecules that act to restrict, organize, and/or regulate the subcellular distribution of other signaling molecules (Pawson and Scott, 1997). For example, a paradigm class of proteins with such functions are the AKAPs (A-kinase anchor proteins), known to contain the localization and activation of protein kinase A to different subcellular compartments, thereby limiting the spurious activation of this potent kinase (Colledge and Scott, 1999).

Indeed, several lines of evidence now suggest that the caveolins might also act as scaffolding proteins by directly interacting with and modulating the activity of caveolae-localized signaling molecules. The first evidence of such function was provided in vitro, where it was shown that a 20-amino acid peptide derived from caveolin-1 (residues 82–101) was a potent inhibitor of heterotrimeric G-proteins in GTP hydrolysis assays (Li et al., 1995). Subsequent work has confirmed the selectivity of this region for binding to and modulating the activity of not only G-proteins but also a host of other signaling proteins (see Table 2 for a complete list) including H-Ras, Src family tyrosine kinases, PKC isoforms, EGF-R, Neu, and eNOS (Engelman et al., 1998c; Okamoto et al., 1998; Smart et al., 1999). Because this region of the caveolin molecule seems to be largely responsible for many of the molecule's functional effects, it has been termed the caveolin scaffolding domain (CSD) (see Fig. 8).

A peculiarity of the CSD seems to be its capability for broad association with disparate signaling molecules. In an attempt to determine possible binding motifs in these caveolin-associated proteins, a glutathione *S*-transferase-fusion protein containing the CSD was used to screen a peptide phage display library (Couet et al., 1997a). Interestingly, only a select group of peptides showed high-affinity binding to the CSD; compilation of these sequences indicating that they almost invariably matched the following motifs: Φ*X*Φ*XXXX*Φ, Φ*XXXX*Φ*XX*Φ, Φ*X*Φ*XXXX*Φ*XX*Φ, where Φ is an aromatic residue (Phe, Tyr, or Trp) and *X* is any amino acid (Couet et al., 1997a). Furthermore, a search of known caveolin-interacting molecules showed that at least one such motif could be identified in their primary sequence, indicating that these regions might be sites of direct interaction with the caveolin-1. Many such motifs that

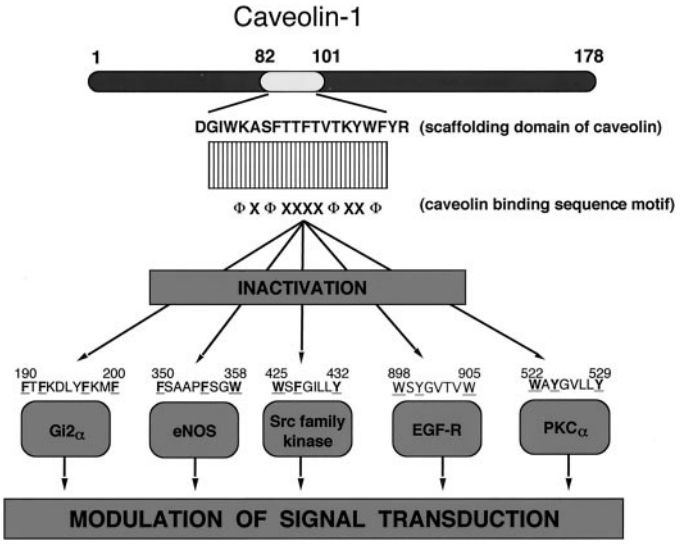

FIG 8. CSD and caveolin binding motif reciprocal interactions. The sequence of the caveolin-scaffolding domain and the caveolin binding sequence motifs within several caveolae-localized signaling molecules are shown. These include G-protein α subunits ($G_{i2}\alpha$), eNOS, Src family tyrosine kinases, receptor tyrosine kinases (EGF-R), and PKC isoforms (PKCα). In most cases, this caveolin interaction is inhibitory, leading to inactivation of the signaling molecules and modulation of downstream signal transduction.

are found in caveolin-interacting molecules are now known as caveolin binding domains (CBDs) (see Fig. 8). However, the presence of a CBD is not an absolute inclusive criteria for caveolin binding, and in fact, many proteins with putative CBDs have not been shown to interact with caveolin. Obviously, the solvent accessibility of such domains is a major factor in the ability of a protein to bind caveolin. Thus far, support for the existence of such domains (namely by demonstrating disrupted caveolin-1 binding upon targeted mutation of a putative CBD) has been provided for only a few signaling molecules (i.e., insulin receptor, eNOS, and GRKs) (Garcia-Cardena et al., 1997; Carman et al., 1999; Nystrom et al., 1999).

Currently, the lack of structural information precludes an assessment of the mechanism by which caveolin-1 and its CSD can recruit and bind to CBD-containing molecules. However, the fact that both the CSD and CBD peptide sequences are enriched in similarly spaced aromatic amino acids (Couet et al., 1997a) suggests that such interactions could occur by a linear juxtaposition of these hydrophobic-hydrophobic interactions. In many respects, the CSD functions as a modular protein domain (akin to SH2, SH3, and WW domains), recognizing motifs in presumably solvent-accessible regions of signaling molecules.

With the exception of a few signaling molecules, interaction with the CSD leads to inhibition of downstream signaling. In the list of caveolin-interacting molecules shown in Table 2, most have been shown to be inhibited by caveolin-1 by either biochemical or cell culture experiments. In the case of tyrosine and serine/

threonine kinases, such functional interaction takes on practical significance. Nearly all enzymes of this class harbor caveolin binding motifs located within the active catalytic domain (namely kinase subdomain IX) (Couet et al., 1997b), and a synthetic peptide corresponding to the CSD is sufficient to inhibit their phosphotransferase activities in vitro. As many of these protein kinases have thus far not been reported to have more potent peptide inhibitors than the CSD, agents that mimic the binding of kinases to caveolins are potentially useful as protein kinase inhibitors and as lead compounds in drug development.

3. Caveolin-2 and -3 As Signaling Modulators. Primarily due to the overwhelming focus on the archetypal caveolin-1, the capacity of the other members of the caveolin family to act as scaffolding proteins remains unknown. Most of what is known about these proteins is by analogy with Cav-1. A glance at the protein alignment in Fig. 3 illustrates that Cav-1 and Cav-3 have highly homologous CSDs (especially in the spacing of the aromatic residues alluded to above), whereas Cav-2 is largely divergent. Therefore, it can be surmised that in muscle cells, where Cav-3 is selectively expressed, it can act as an effective scaffolding protein in the absence of other caveolins. Indeed, the few experiments that have been conducted with Cav-3 and its CSD seem to corroborate this idea (Li et al., 1995; Yamamoto et al., 1998; Razani et al., 1999). At present however, the dearth of data on the functions of caveolin-2 preclude its implication in any known signaling pathway.

4. Signaling Spotlight: Modulation of Endothelial Nitric-Oxide Synthase Function. Probably the best studied of the signaling molecules that are modulated by caveolin is eNOS. Acylation of eNOS serves to target it to lipid-raft domains of the plasma membrane and the Golgi apparatus (Garcia-Cardena et al., 1996b; Shaul et al., 1996; Sowa et al., 2001; Fulton et al., 2002) (Fig. 9). eNOS localized to lipid rafts/caveolae, but not interacting with Cav-1, has optimal enzymatic function; however, interaction with Cav-1 inhibits eNOS function (Garcia-Cardena et al., 1996a; Liu et al., 1996a; Michel et al., 1997a; Sowa et al., 2001). The region of Cav-1 responsible for the inhibition of eNOS has been mapped to the CSD (Garcia-Cardena et al., 1997; Ju et al., 1997). Point mutations in the CBD of eNOS results in the loss of Cav-1 binding without the loss of eNOS enzymatic activity (Garcia-Cardena et al., 1997). Thus a model has been proposed in which eNOS is targeted to caveolae/lipid rafts where the CSD/CBD interaction serves to maintain eNOS inhibition until the CSD/CBD interaction is disrupted due to an increase in cytosolic calcium followed by calcium-calmodulin/eNOS interaction (Fig. 9) (Garcia-Cardena et al., 1997; Michel et al., 1997a, b).

The physiological significance of the Cav-1/eNOS interaction was recently demonstrated by Sessa and colleagues (Bucci et al., 2000). Using ex vivo aortic rings, they were able to show that a cell-permeable peptide containing the CSD potently inhibits acetylcholine (Ach)-induced NO production and vasodilation. The CSD was also able to ameliorate the NO-mediated vascular

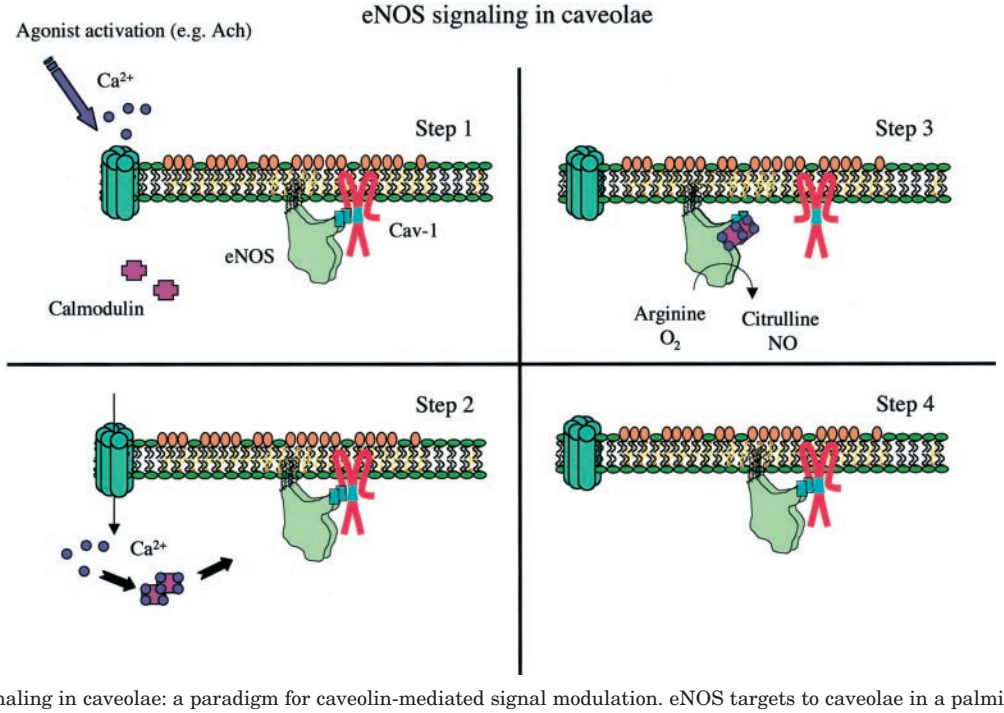

FIG 9. eNOS signaling in caveolae: a paradigm for caveolin-mediated signal modulation. eNOS targets to caveolae in a palmitoylation-dependent manner. Although localization to caveolae is pivotal for maximal eNOS activity, caveolin-1 can inhibit this activity by directly interacting with eNOS. Depicted in the figure is the cycle of eNOS activation and inhibition in caveolae: step 1, Cav-1 provides tonic inhibition of eNOS activity; step 2, agonist activation by Ach initiates an influx of calcium ions that bind to and activate calmodulin; step 3, calcium-activated calmodulin binds to eNOS thereby relieving its tonic inhibition by Cav-1 and NO is produced; and step 4, Cav-1 binds to eNOS again, completing the cycle.

compromise in mice treated with pro-inflammatory agents (Bucci et al., 2000).

The emerging view is that caveolins may function as general negative regulators to inhibit the basal activity of many signaling proteins. Upon activation of a given signaling pathway, caveolin-mediated inhibition is released thus increasing the signal propagation (Okamoto et al., 1998; Smart et al., 1999; Ostrom et al., 2000b). The logical consequence of this view is the implication of Cav-1 as a tumor suppressor based on the capacity of Cav-1 to inhibit the functional activity of several proto-oncogenes (see below).

5. Signaling Spotlight: the Dynamic Relationship of G-Protein-Coupled Receptors and Caveolae. When G-protein coupled receptors (GPCRs) and G-proteins were first isolated in low buoyant-density sucrose gradients, it was postulated that caveolae may serve as platforms for congregating these receptors with their downstream effectors (Lisanti et al., 1994a, b). Since that time, a more dynamic interaction between receptors and caveolae has emerged by comparing the caveolar localization of proteins before and after agonist stimulation.

It has been shown that β adrenergic receptors can localize to caveolae (Schwencke et al., 1999; Ostrom et al., 2000b; Rybin et al., 2000). Further work has revealed that β_2 adrenergic receptors are highly enriched in caveolae at rest but exit caveolae upon adrenergic stimulation (Ostrom et al., 2001), where they are thought to be targeted to clathrin-coated pits for internalization. Importantly, the downstream effector, adenylyl cyclase type 6, also localizes to caveolae where its proximity to activated β_2 adrenergic receptors may facilitate downstream signaling before receptor migration (Ostrom et al., 2000b, 2001). Adrenergic stimulation failed to cause the translocation of β_1 adrenergic receptors out of caveolae, however (Ostrom et al., 2000b). The observed higher cAMP response to β_1 adrenergic receptor-selective activation is hypothesized to be due to the retention of the β_1 adrenergic receptor in caveolae where it remains in proximity to adenylyl cyclase type 6 (Ostrom et al., 2001). It is also thought that the localization of many endogenous β_1 adrenergic receptors outside of caveolae versus the complete enrichment of β_2 adrenergic receptors in caveolae may explain signaling differences as well (Ostrom et al., 2000a, 2001; Steinberg 2001).

As opposed to trafficking out of caveolae, the β_1 and β_2 bradykinin receptors both translocate into caveolae upon agonist stimulation (Sabourin et al., 2002). Their ultimate cellular fate, however, is different. The β_2 bradykinin receptor moves into caveolae and then is internalized, whereas the β_1 bradykinin receptor is not internalized. This difference in internalization may explain the previously observed difference in the phosphorylation of the cytoplasmic tail of β_2 bradykinin receptor versus the lack of phosphorylation of β_1 bradykinin receptor cytoplasmic tails. Further functional studies will be necessary to determine how the localization of β_1 and β_2 bradykinin receptors effects their signaling capability. Another receptor found to translocate into caveolae upon agonist activation is the M_2-muscarinic acetylcholine receptor (Feron et al., 1997). It is thought that the movement of the M_2-muscarinic acetylcholine receptor into caveolae may enhance its ability to activate eNOS, which alters parasympathetic regulation.

The colocalization of GPCRs with their effectors in caveolae certainly suggests a role for caveolae in signal transduction. The movement of GPCRs into or out of caveolae upon agonist stimulation further reinforces the view that caveolae are dynamic signaling domains.

D. Oncogenes and Tumorigenesis

1. Caveolae / Caveolins As Targets of Oncogenes. The association between caveolins and cancer dates back to the discovery of caveolin-1 as a predominant phosphoprotein in v-*src*-transformed embryonic chicken fibroblasts (Glenney, 1989). However, follow-up work on the role of caveolae/caveolins in tumorigenesis was not conducted until it was reported that the oncogenic transformation of NIH 3T3 cells results in transcriptional down-regulation of Cav-1 and ablation of morphologically identifiable caveolae (Koleske et al., 1995). This observation along with several subsequent studies in other tumor-derived cell lines and primary carcinomas (Engelman et al., 1998b; Lee et al., 1998; Razani et al., 2000a) emphasizes that regardless of the oncogenic process, caveolae and Cav-1 seem to be important targets in tumorigenesis.

One of the hallmarks of cellular transformation is the activation or amplification of proto-oncogenes, a process which imparts survival/growth advantages to the cell (Macleod and Jacks, 1999). The fact that many tumor cells show down-regulation of caveolin-1 could indicate that Cav-1 is a direct target of the activated oncogenes in these cells. Indeed, this was first definitively demonstrated for the activated form of H-Ras (G12V mutant): stable expression of H-Ras(G12V) in NIH 3T3 cells leads to the down-regulation of Cav-1 (Koleske et al., 1995), whereas treatment of these cells with an inhibitor of the Ras-p42/44 MAP kinase cascade reverts Cav-1 expression to wild-type levels (Engelman et al., 1997). Since these initial observations, a growing list of other oncogenes have also been shown to down-regulate Cav-1 protein expression at the transcriptional level (Table 3) (Koleske et al., 1995; Engelman et al., 1997, 1998b; Razani et al., 2000a; Park et al., 2001). In all but a few cases, the mechanism of this transcriptional suppression has not been elucidated, a situation requiring detailed dissection of the caveolin-1 promoter. Exceptions include the finding of functional c-Myc-repressive and p53-responsive elements in the Cav-1 promoter, thereby explaining the mechanism of Cav-1 down-regulation by c-*myc* and the human papilloma virus oncogene E6, an

oncogene that targets p53 for degradation (Bist et al., 1997; Razani et al., 2000a; Park et al., 2001).

2. The Caveolins As Tumor Suppressors. Because caveolin-1 has been shown to act as a scaffolding protein with a capacity to inhibit various signaling pathways, it can be surmised that the function of certain proto-oncogenes and/or activated oncogenes might be similarly regulated. This might provide a teleological reason as to why Cav-1 is a common target among several activated oncogenes (see Table 3). Overexpression experiments indicate that Cav-1 is indeed a potent inhibitor of some of these pro-proliferative pathways. For example, Cav-1 can interact with and suppress the function of the EGF receptor and several members of the Ras-p42/44 MAP kinase cascade (Fig. 10) (Couet et al., 1997b; Engelman et al., 1998a). Furthermore, antisense-mediated down-regulation of caveolin-1 in NIH 3T3 is sufficient to hyperactivate the Ras-p42/44 MAP kinase cascade and leads to cellular transformation (Galbiati et al., 1998). Interestingly, ablation of caveolin-1 expression in *Caenorhabditis elegans* via RNAi leads to hyperactivation of the meiotic cell cycle, a phenotype strikingly similar to uncontrolled Ras signaling (Scheel et al., 1999). Cav-1 has been found to have similar inhibitory effects on several other oncogenes, namely c-Neu and c-Myc (Fig. 10) (Engelman et al., 1998b; Park et al., 2001).

The decision to survive and proliferate or to commit to apoptosis is essential for normal embryonic development, for maintaining tissue homeostasis, and as a safeguard against tumorigenesis in the face of cellular/genomic damage. Indeed, there is increasing evidence to suggest that the caveolins and caveolae may also be involved in shifting the tightly regulated balance from anti-apoptotic to pro-apoptotic signaling. Cav-1 has been shown to interact and inactivate a number of signaling molecules involved in survival/proliferation, such as the PDGF receptor and phosphatidylinositol 3-kinase (Fig. 10) (Liu et al., 1996b; Yamamoto et al., 1999; Zundel et al., 2000). However, aside from this seemingly pleiotropic suppression of plasma-membrane-initiated pro-survival pathways, caveolae and the caveolins appear to have a highly specialized role as well. Ceramide is an essential factor for the commitment to apoptosis induced by several cellular stressors (Basu and Kolesnick, 1998). Interestingly, sphingomyelin, the precursor to ceramide generation, is one of the most abundant lipids in caveolae, and sphingomyelinase, the ceramide-generating enzyme, has been localized to caveolae microdomains (Liu and Anderson, 1995). Furthermore, overexpression of Cav-1 sensitizes cells to ceramide-induced cell death via a phosphatidylinositol 3-kinase-dependent mechanism (Zundel et al., 2000). Therefore, the production of ceramide and its downstream actions seem to depend on caveolar localization and caveolin-1 regulation. In support of these results, overexpression of Cav-1 sensitizes cells toward apoptotic stimuli, whereas antisense-mediated down-regulation of Cav-1 imparts resistance to apoptosis (Liu et al., 2001).

TABLE 3
Oncogenes that transcriptionally suppress Cav-1 expression

Oncogene	Reference(s)
H-ras^{G12V}	Koleske et al., 1995; Engelman et al., 1997
v-*abl*	Koleske et al., 1995; Engelman et al., 1997
mTAg	Koleske et al., 1995
bcr-abl	Koleske et al., 1995
crk1	Koleske et al., 1995
c-*src*	Engelman et al., 1998b; Ko et al., 1998
c-*neu*	Engelman et al., 1998b
HPV E6	Razani et al., 2000a
c-*myc*	Timme et al., 2000; Park et al., 2001

The evidence in support of caveolin-1 as a tumor suppressor has for the most part been elucidated in cell culture systems, with substantially less research conducted in vivo. Intriguingly, it is known that the caveolin-1 and -2 genetic loci are situated close to a region of chromosome 7 (7q31.1) that is frequently deleted in numerous forms of human cancer (Engelman et al., 1998d). Below, we will expand on this observation and critically evaluate its relevance.

3. Relevance to Human Cancers. LOH (loss of heterozygosity) analysis has determined that the q31 region of human chromosome 7 is a region of high deletion frequency in many types of human epithelial tumors, including human primary breast (Zenklusen et al., 1994a), prostate (Zenklusen et al., 1994b; Jenkins et al., 1998), ovarian (Kerr et al., 1996), colon (Zenklusen et al., 1995a), and renal cell carcinomas (Shridhar et al., 1997). More specifically, most of these deletions have been found to be normally distributed around the D7S522 CA-repeat microsatellite, a marker that maps to the 7q31.1 region of the human chromosome (Zenklusen et al., 1994b, 1995a,b; Lin et al., 1996). The frequent involvement of this region in different types of cancers is highly suggestive for the presence of a previously uncharacterized tumor suppressor gene (Zenklusen et al., 1994b; Jenkins et al., 1998); however, candidate genes mapping to this loci had never been described.

In an unrelated attempt to map the human caveolin-1 and -2 loci, Lisanti and colleagues made the interesting observation that both genes are colocalized to the q31.1 region of human chromosome 7 (Engelman et al., 1998d). Further dissection of the loci revealed that D7S522 was actually the closest microsatellite marker of any tested, located ~67 kb upstream of the CAV-2 gene, which in turn was followed ~19 kb later by the CAV-1 gene (Fig. 11) (Engelman et al., 1998d, 1999). At the time of these reports, the caveolins were the first genes to be so closely localized to this "tumor suppressor" locus; given its involvement in proliferative/apoptotic pathways, Cav-1 was proposed to be the candidate tumor suppressor hypothesized to be located in this region (Engelman et al., 1998c, 1999).

In support of this notion, several recent reports have also demonstrated defects in the caveolin genetic locus in primary tumors. The caveolin-1 promoter region is

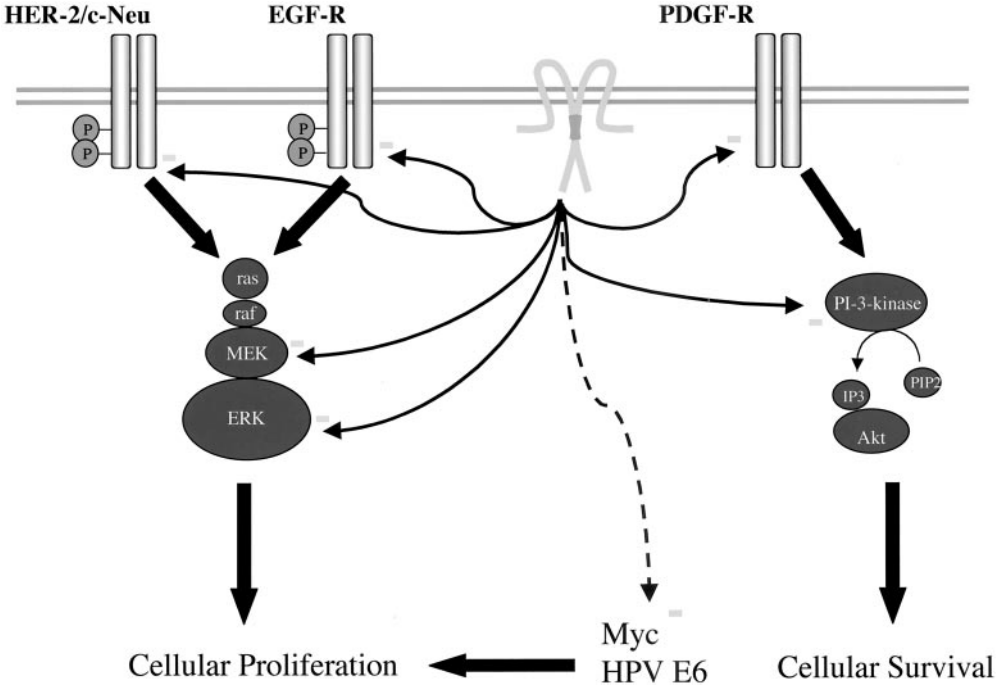

FIG 10. Caveolin-1 negatively regulates signaling along several pro-proliferative and anti-apoptotic pathways. Consistent with a tumor-suppressor role, Cav-1 can potently inhibit signaling originating from certain receptor tyrosine kinases (HER-2/c-Neu and EGF-R) and some of their downstream components (including the Ras-p42/44 MAP kinase cascade). In addition, Cav-1 has been shown to facilitate apoptotic signaling by shutting down certain members of the pro-survival phosphatidylinositol 3-kinase (PI-3-kinase)/Akt pathway. The other inhibitory functions of Cav-1 (e.g., abrogation of Myc- or HPV E6-mediated transformation) are less well understood.

hypermethylated in breast cancer-derived cell lines and prostate cancer-derived tumor samples (Engelman et al., 1999; Cui et al., 2001), indicating that transcriptional silencing might be a mechanism of abrogating caveolin function. In addition, a recent report found that the CAV-1 gene is mutated in up to 16% of human breast cancer samples examined, with the majority of these being invasive carcinomas (Hayashi et al., 2001). Also, a Cav-1 cDNA harboring this point mutation (P132L) is sufficient to transform NIH 3T3 cells (Hayashi et al., 2001). Since an analogous mutation in the caveolin-3 gene (P104L) (see Fig. 3 for comparison) has been implicated in several patients with an autosomal dominant limb-girdle muscular dystrophy (LGMD-1C—described below) (Minetti et al., 1998), it is likely that the P132L mutation behaves in a dominant-negative fashion.

The role of Cav-1 as a tumor suppressor remains controversial. With the availability of highly informative genomic sequences and previously detailed physical mapping of the 7q31 region, Zenklusen and colleagues (2001) recently attempted to definitively identify the culprit tumor suppressor gene in this locus. They suggest instead that a gene called ST7 is the long sought tumor suppressor gene, having identified ST7 mutations (premature stop codons) in primary tumors and cell lines. In addition, they show that recombinant expression of ST7 in a human tumor cell line can inhibit tumorigenicity in nude mice. Unfortunately, with no analysis of the caveolin sequences, Zenklusen and colleagues dismiss the idea that caveolin-1 is a functional tumor suppressor, stating previous studies have found no mutations or promoter methylation associated with

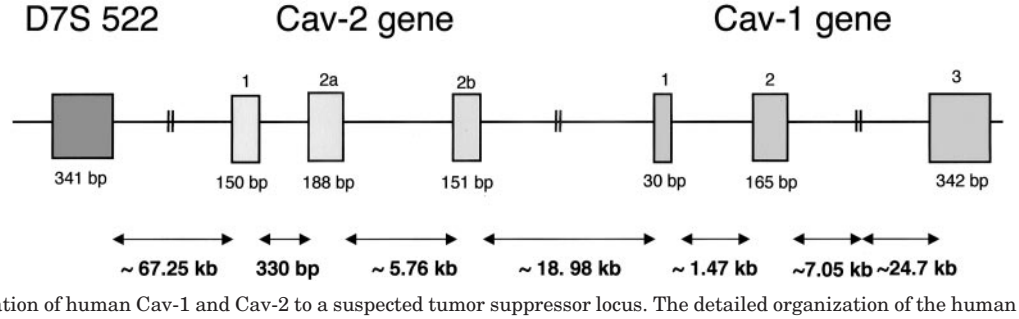

FIG 11. Localization of human Cav-1 and Cav-2 to a suspected tumor suppressor locus. The detailed organization of the human Cav-1/2 locus (with all exons and introns) and its relationship to D7S522 is shown. Note that the marker D7S522 is located ~67 kb upstream of Cav-2, and Cav-2 is located ~19 kb upstream of Cav-1. Given that D7S522 is at the center of the smallest common deleted region, and both Cav-1 and Cav-2 are <100 kb away, these genes are one of the closest functionally relevant genes to this frequently deleted region (see text for recent reports on the ST7 gene).

these genes (CAV-1 and CAV-2). As described above, this is clearly not true. It is very possible that the observed LOH in the 7q31 region could still be primarily due to Cav-1, with some contribution from the newly identified ST7. Undoubtedly, this issue requires further analysis.

E. Specialized Functions: Caveolin-3 and Muscle Cells

Cav-3 is 65% identical and 85% similar to Cav-1 (see Fig. 3). Given that Cav-3 is the only caveolin expressed in striated muscle cells, it might be assumed that Cav-3 performs analogous functions to those performed in other tissues by Cav-1. Akin to Cav-1-generated caveolae, the Cav-3-generated caveolae of striated muscle have been shown to be enriched in a variety of signaling molecules: eNOS; type B atrial natriuretic factor receptor; m2 muscarinic acetylcholine receptor; PKCα, PKCδ, PKCϵ; Erb 4; adenosine A(1) receptors; Gαs, Gαi, Gαq; α_1, β_1, β_2 adrenergic receptor; adenylyl cyclase, Src family kinases (Feron et al., 1996, 1997; Song et al., 1996b; Venema et al., 1997; Rybin et al., 1999, 2000; Lasley et al., 2000).

However, Cav-3 and the caveolae that they form appear to have a specialized role, in addition to signaling compartmentalization, in skeletal muscle tissue. Muscle caveolae contain a portion of the cellular dystrophin-glycoprotein (DG) complexes. (Song et al., 1996b). Although not an intrinsic member of the DG complex (Crosbie et al., 1998), Cav-3 has been shown to coimmunoprecipitate with DG complex proteins (i.e., α-sarcoglyan, β-dystroglycan, and dystrophin (Song et al., 1996b; Sotgia et al., 2000), as well as to interact with other proteins that associate with the DG complex (i.e., nNOS) (Brenman et al., 1995). Cav-3 possesses a unique WW domain that binds to the PPXY motif within β-dystroglycan (Sotgia et al., 2000). Interestingly, dystrophin also interacts with β-dystroglycan via this motif, thus suggesting a role for competitive binding between Cav-3 and dystrophin. These molecular studies thus identify Cav-3 as a DG complex-interacting protein (see Fig. 12 for an overview of the DG complex).

Initial EM analysis of caveolae in striated muscle led to the hypothesis that the T-tubule system forms via the coalescence and fusion of numerous caveolae (Ishikawa 1968; Parton et al., 1997). After the identification of the Cav-3 protein and anti-Cav-3 antibody generation (Song et al., 1996b; Tang et al., 1996), a multitude of experimental evidence has emerged that supports the role of Cav-3/caveolae in T-tubule development. Transcripts of Cav-3 mRNA are first detectable in vivo at day 10 of gestation in developing somites and heart (Biederer et al., 2000), well before T-tubule maturation. Caveolae and Cav-3 were shown to be transiently associated with the skeletal muscle T-tubule system during its developmental stages (Parton et al., 1997) and are localized to sarcolemmal caveolae in fully differentiated skeletal muscle (Song et al., 1996b). The T-tubule system is an elaborate network of membrane invaginations that penetrate the depth of muscle cells and allows for the rapid rise in intracellular Ca^{2+} upon membrane depolarization resulting in muscle contraction (Flucher et al., 1994). In vitro experiments corroborate these studies, because antisense-mediated down-regulation of Cav-3 in the skeletal myoblast cellline C2C12 is sufficient to abrogate myoblast fusion and myotube formation (Galbiati et al., 1999a).

The importance of Cav-3 for proper skeletal muscle function came to light when Minetti, Lisanti, and colleagues identified two distinct mutations in the Cav-3 gene that each result in an autosomal dominant form of limb-girdle muscular dystrophy (LGMD-1C) (Minetti et al., 1998). Patients with this disease present with calf hypertrophy, muscle cramps, and mild-to-moderate proximal muscle weakness. Further analysis shows elevated serum creatine kinase levels, typical of a pathological muscle phenotype. Histological analysis reveals only moderate myopathic changes but a near complete loss of Cav-3 protein expression. In vitro studies in which the mutant Cav-3 proteins were overexpressed in a heterologous cell system demonstrated that these LGMD-1C mutants interact with normal Cav-3 proteins to form unstable aggregates, which undergo ubiquitination and proteasomal degradation (Galbiati et al., 1999b). The analysis of other patients with mutations in Cav-3 and mouse models of caveolin-3-opathies has yielded an even greater understanding of Cav-3 function in muscle (see relevant headings below).

F. Emerging Functions: Caveolins and Lipid Droplets

Lipid droplets, albeit traditionally associated with adipocytes, are found to varying extents in almost all cells (Londos et al., 1999). They are thought to consist of a core of neutral lipids (i.e., triglycerides and cholesterol esters) surrounded by a phospholipid monolayer derived from the ER. Several proteins, including the highly abundant perilipins (present in adipocytes and steroidogenic cells) and adipocyte differentiation-related protein (ADRP; present in most other lipid droplet-containing cells), coat this lipid core and maintain its overall integrity (Londos et al., 1999).

Recently, several independent investigators have shown that the caveolins can be redirected to lipid droplets under conditions where they are localized to the ER (Ostermeyer et al., 2001; Pol et al., 2001). Parton and colleagues showed that truncated versions of caveolin-1, -2, and -3 mis-localize to intracellular cholesterol-rich vesicles, which they later identified as lipid droplets (Roy et al., 1999; Pol et al., 2001). In a more direct experiment, Brown and colleagues tagged the caveolin-1 protein with the ER-retrieval sequence, KKSL, and showed caveolin-1 accumulation in lipid droplets (Ostermeyer et al., 2001). The wild-type untagged Cav-1 protein can be similarly redirected by treating cells with brefeldin A, which collapses the Golgi/ER compartments

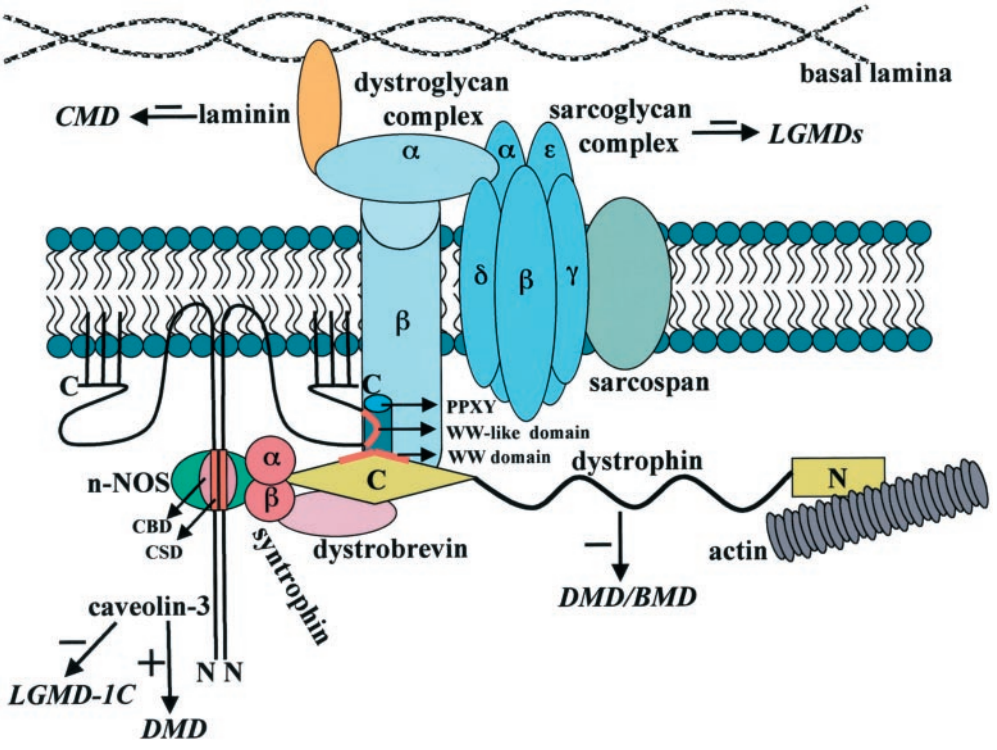

FIG 12. The DG complex and its associated proteins at the muscle cell plasma membrane. The DG complex links the extracellular matrix to cytoskeletal elements. β-Dystroglycan is the only component of the complex that directly interacts with dystrophin. The WW domain within dystrophin and the WW-like domain within caveolin-3 both bind to the PPXY motif within β-dystroglycan. Up-regulation of Cav-3 (as seen in Duchenne muscular dystrophy patients, in *mdx* mice, and in Cav-3 over-producing transgenic mice) can displace dystrophin from the plasma membrane, inducing destabilization and, eventually, degradation of the protein. Arrows indicate diseases caused by the absence (−) or up-regulation (+) of the indicated gene. DMD, Duchenne muscular dystrophy; BMD, Becker muscular dystrophy; CMD, congenital muscular dystrophy; LGMDs, multiple forms of limb-girdle muscular dystrophy; LGMD-1C, limb-girdle muscular dystrophy, type 1C; nNOS, neuronal form of nitric oxide synthase; CBD, caveolin binding motif. Modified from Galbiati et al., 2001c.

or by incubation of cells with the free fatty acid, oleate (Ostermeyer et al., 2001; Pol et al., 2001). Interestingly, overexpression of the β-isoform (not full-length version) of caveolin-2 leads to its constitutive localization to lipid droplets (Fujimoto et al., 2001). Taken together, these data suggest that although the caveolins are not normally associated with internal lipid compartments, they can certainly traffic to these locations under certain conditions. As such, the caveolins are the first known integral membrane protein components of lipid droplets. Although the physiological relevance of this lipid droplet localization is not known, these findings could be extremely valuable in the understanding of caveolin/caveolae functions in adipocytes.

Based purely on ultrastructural comparisons and tissue expression profiles, the adipocyte seems to have the highest concentrations of caveolae and the highest levels of caveolin-1 and -2, i.e., more than any other cell type (Scherer et al., 1994). Indeed, electron micrographs of adipocytes dating back to 1963 show that caveolae account for ~30% of the surface area of the adipocyte plasma membrane (Napolitano 1963; Fan et al., 1983). Furthermore, in 3T3-L1 cells, a widely used model system for studying adipogenesis, the number of caveolae increases ~9-fold, and caveolin-1 and -2 expression increases ~20-fold during differentiation from the fibroblastic to the adipocyte state (Fan et al., 1983; Scherer et al., 1994).

The uptake and storage of fatty acids as triglycerides is a major function of adipocytes. As the flux of fatty acids into primary adipocytes and 3T3-L1 cells follows saturable kinetics, facilitated membrane transport has been proposed as the uptake mechanism (Abumrad et al., 1984; Zhou et al., 1992). Interestingly, labeling of membrane proteins with photoreactive long-chain fatty acids identifies caveolin-1 as the major fatty acid binding protein in adipocytes (Gerber et al., 1993; Trigatti et al., 1999). Together with the association of the caveolins with lipid droplets, it is entirely possible that caveolae and the caveolins act as portals for the uptake and transport of fatty acids to lipid droplets. In addition to Cav-1, several other proteins have been proposed to mediate this function (Bernlohr et al., 1999). A true understanding of lipogenic processes will require comparisons between this disparate group of fatty acid binding proteins.

VI. Animal Models in the Study of Caveolae and Caveolins

Although interest in caveolae and the caveolins has been steadily increasing over the last 10 years, much of

the research has been limited to biochemical or cell culture analyses. Despite the successes of this approach (namely the implication of caveolae/caveolins in endocytosis, cholesterol trafficking, signal transduction, tumorigenesis, and other processes), it is not clear which of the pleiotropic functions of caveolae are predominant in vivo and thus physiologically relevant. In this regard, the recent series of reports describing the various phenotypes of mice deficient in Cav-1, -2, or -3 are extremely enlightening (Drab et al., 2001; Galbiati et al., 2001a; Razani et al., 2001a, 2002b).

Perhaps the most important initial observation made in these mice is that in the absence of Cav-1 or Cav-3, but not Cav-2, there is a concomitant loss of morphologically identifiable caveolae in tissues expressing those proteins. Therefore, the Cav-1 and -3 null mice are essentially tissue-specific caveolae-deficient mice, thus enabling the first controlled study of such vesicles in vivo. We will now discuss the previously proposed functions of caveolae in the context of these caveolin-deficient animal models and the relevance of these mouse models to the understanding of human disease.

A. Studies of Caveolin-1-Deficient Mice

The initial characterization of mice with a disruption of the caveolin-1 (Cav-1) locus was reported simultaneously by two groups (Drab et al., 2001; Razani et al., 2001a). At first glance, it is surprising that mice lacking Cav-1 and caveolae would display no "overt" phenotypic abnormalities. After all, it is hard to imagine that certain tissues like the lung or adipose (where ~30–70% of the membrane is composed of these organelles) can function in their absence. However, more detailed histological and functional analysis reveals a number of interesting abnormalities, which we will discuss below (for a summary, see Table 4).

1. Caveolin-1 and Caveolae Biogenesis. As discussed above, several overexpression studies point to Cav-1 as a mediator of caveolae formation (Fra et al., 1995b; Engelman et al., 1997; Galbiati et al., 1998; Li et al., 1998). In this regard, the generation of Cav-1 null mice served as an important proof of principal. Indeed, there is a complete ablation of morphologically identifiable caveolae in Cav-1 null tissues (endothelial and adipose) and primary cells derived from Cav-1 null mice, firmly establishing that caveolin-1 is required for caveolar invagination (Fig. 13A) (Drab et al., 2001; Razani et al., 2001a).

Unfortunately, although it is clear that caveolae formation is dependent on Cav-1 expression, we still lack an understanding of the underlying process. For example, is the loss of caveolae simply a direct result of an absence of Cav-1 or is it secondary to perturbations in the cholesterol content of the plasma membrane? The present literature indicates an important role for caveolin-1 in intracellular cholesterol transport (Fielding and Fielding, 2000). It will be interesting to see if Cav-1 null cells still maintain the normal composition of cholesterol/lipids in the plasma membrane and the extent by which their lipid raft network is perturbed. Recent work by Sotgia et al. (2002) demonstrates that Cav-1 null cells and tissues are clearly not wild type in behavior, exhibiting defects in the sorting of GPI-anchored and lipid-modified proteins. Further work in this area will be rewarding not only in relation to caveolar biogenesis, but in deciphering the role of caveolins in intracellular lipid/protein trafficking.

2. Interactions of Caveolin-1 with the Other Caveolins. As caveolin-1 is known to have very intricate interactions with its closely related family member, Cav-2, Cav-1 null mice also provided the opportunity to dissect this relationship in vivo. Intriguingly, in all Cav-1 null tissues examined, the expression of Cav-2 is severely reduced by ~90 to 95% (Drab et al., 2001; Razani et al., 2001a).

As Cav-2 mRNA levels remain unperturbed, this reduction cannot be transcriptional. Using mouse embryonic fibroblasts (MEFs), Razani et al. (2001a) discovered that in the absence of Cav-1, Cav-2 is destabilized and degraded by the proteasomal pathway. In Cav-1 null cells, any residual Cav-2 protein is found in the Golgi apparatus; however, reintroduction of Cav-1 in these cells rescues both the expression and plasma membrane localization of Cav-2. The same effect can be reproduced by the addition of proteasomal (but not lysosomal) inhibitors, suggesting that Cav-2 likely misfolds at some point during ER/Golgi transport and is targeted for degradation. The fact that at steady state low levels of Cav-2 are localized to the Golgi most likely reflects leakage from and/or inefficiencies in the ER quality control apparatus.

3. Caveolin-1 and Cellular Proliferation. Based on several lines of evidence discussed above (namely the inhibitory action of Cav-1 on pro-proliferative/anti-apoptotic pathways), caveolin-1 has been considered a candidate tumor suppressor. Thus, it would be predicted that Cav-1-deficient cells would exhibit derangements in proliferation and/or growth. Indeed, an analysis of the growth properties of cultured primary MEFs reveals that Cav-1 null cells display a more active cell cycle profile than their wild-type counterparts (~30% increase in the S-phase) and attain nearly 3-fold higher monolayer densities over a 10-day period (Razani et al., 2001a). A number of prior studies support a role for Cav-1 in the suppression of the Ras-MAP kinase pathway (Couet et al., 1997b; Engelman et al., 1998a; Galbiati et al., 1998; Scheel et al., 1999). Therefore, of the disinhibited signaling pathways that might be driving this proliferation, the p42/44 MAP kinase cascade was thought to be the most likely. Surprisingly, Cav-1 null MEFs show no hyperactivation of this pathway (under both baseline and stimulated conditions) (Razani et al., 2001a). Thus, to understand the basis for this hyperpro-

TABLE 4
Phenotypic analysis of caveolin-deficient mice

Cav-1 Knock-out	Cav-2 Knock-out	Cav-3 Knock-out
Loss of caveolae In fibroblasts, endothelia, and adipocytes	Caveolae intact	Loss of caveolae In skeletal muscle fibers
Near total deficiency of Cav-2 Rescued by Cav-1 expression	Partial reduction in Cav-1 in certain tissues	Myopathic changes (mild-to-moderate) Variability in muscle fiber size Presence of necrotic fibers
MEFs Defects in caveolar endocytosis Hyperproliferative phenotype Decreases in G_o/G_1-phase Increases in S-phase		Dystrophin-glycoprotein complex Mislocalized No longer targeted to lipid rafts
Lung abnormalities Hypercellularity Thickened alveolar septa Exercise intolerance Defects in caveolar endocytosis	Lung abnormalities (identical to Cav-1 KO) Thickened alveolar septa Exercise intolerance	T-tubule abnormalities Mislocalization of T-tubule markers Ryanodine receptor DHPR-1α T-tubules (ferrocyanate staining) Dilated/swollen Longitudinally oriented Run in irregular directions
Decreased Vascular Tone Secondary to eNOS activation		
Adipocyte/lipid abnormalities Resistant to diet-induced obesity Atrophic fat pads Reduction in adipose cell diameter Hypercellular/poorly differentiated Hypertriglyceridemia		
Clinical relevance Restrictive lung diseases Cancer Dominant negative mutation in human breast cancer Cav-1 (P132L) (sporadic) Obesity, hyperlipidemias, diabetes	Clinical relevance Restrictive lung diseases Important animal model in conjunction with Cav-1 KO	Clinical relevance Muscular dystrophy Dominant negative mutations in LGMD-1C in humans Cav-3 (P104L) and others (autosomal dominant)

liferation, future studies will need to take into account a broader array of cell cycle regulators.

As an adjunct to cell culture experiments, an understanding of caveolin function in tumorigenesis will require whole animal studies. Cav-1-deficient mice do not exhibit accelerated tumor development over their wild-type counterparts even in older mice (up to 1 year of age) (Razani et al., 2001a). However, it is possible that the loss of Cav-1 in conjunction with another tumor suppressor can act synergistically in tumorigenesis. Such questions can be addressed by crossing Cav-1 null mice with one or more of the many tumor-prone mice currently available.

4. Caveolin-1 and Endocytosis. A large body of work suggests that caveolae are the predominant route by which certain molecules (e.g., cholera and tetanus toxins, albumin, GPI-anchored proteins), viruses, and even bacteria are internalized by a cell (Montesano et al., 1982; Anderson et al., 1992; Parton et al., 1994; Schnitzer et al., 1994; Anderson, 1996; Shin et al., 2000; Stang et al., 1997). As Cav-1-deficient mice also lack caveolae, especially in tissues with central roles in endocytic processes (e.g., the endothelium), it is possible to rigorously examine the actual contribution of caveolae in this setting.

Two recent studies focusing on the uptake of albumin in the Cav-1 null setting (conducted in vitro and in vivo) have provided support for caveolae-mediated endocytosis (Razani et al., 2001a; Schubert et al., 2001). Cav-1 null MEFs incubated with fluorophore-conjugated albumin are entirely defective in membrane labeling and internalization, an effect that can be reversed by transfection of the Cav-1 cDNA. In contrast, the uptake of fluorophore-conjugated transferrin, a clathrin-mediated event, remained unaffected in these cells (Razani et al., 2001a). Infusion of gold-conjugated albumin into the pulmonary vasculature of wild-type and Cav-1 null mice and subsequent electron microscopy reveals that in the absence of caveolae, the albumin load remains strictly luminal (Schubert et al., 2001). Similar results were obtained in a more quantitative assay of endocytosis using radioiodinated albumin (Schubert et al., 2001). Based on this evidence, caveolae appear to be the predominant pathway by which a major serum protein is endocytosed.

However, as albumin makes up ~60% of the serum protein mass and serves as the major serum-transport protein for numerous endogenous molecules, it is difficult to reconcile the above data with the viability and

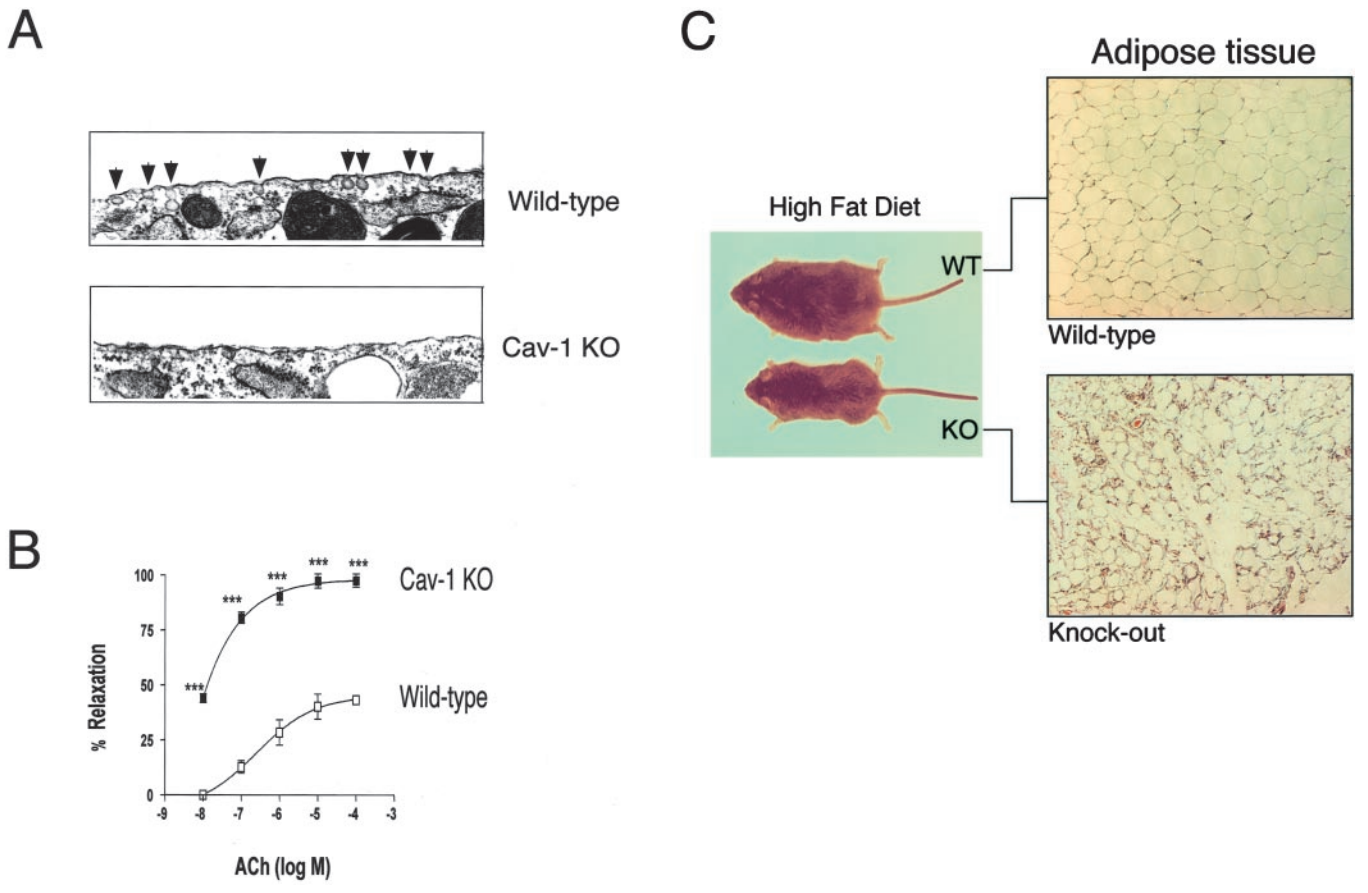

FIG 13. Some important phenotypes of caveolin-1-deficient mice. A, a deficiency in Cav-1 is sufficient to completely disrupt caveolae formation. Cell types thus far examined include endothelial cells, adipocytes, and MEFs. The transmission electron micrographs shown are from near-confluent MEFs, imaged at 16,000× magnification (for ease of view, images shown are further magnified to 43,500×) (Razani et al., 2001a). B, caveolin-1-deficient mice show hyper-responsiveness to endothelium/NO-dependent vasodilation. Note that Ach-induced relaxation (a NO-dependent phenomenon) of the aortic rings was clearly potentiated by the loss of caveolin-1 expression. Concentration-dependent relaxation induced by Ach (expressed as log of molar concentration) in aortas preconstricted with 10 μM phenylephrine from wild-type (WT; □) and Cav-1 null (KO; ■) mice. Points represent mean ± S.E.M of 5 (KO) or 6 (WT) rings from 3 mice each. ★★★, $P < 0.0001$ versus wild-type (Razani et al., 2001a). C, on the left is a photograph of representative wild-type and Cav-1(−/−) mice on a high-fat diet at the end of the study (36 weeks of age). Note that a deficiency in caveolin-1 imparts resistance to diet-induced obesity. Shown on the right is the routine histological analysis (H&E) of the adipose tissue in the same mice. Regardless of the location of the adipose tissue (i.e., subcutaneous, peri-gonadal, or peri-renal/retroperitoneal), Cav-1 null adipocytes have a reduced cell diameter and a poorly differentiated/hypercellular adipose parenchyma (Razani et al., 2001a).

overtly benign presentation of Cav-1-deficient mice. Obviously, other pathways must at least partially compensate for the absence of caveolae in vivo. In fact, when Drab et al. (2001) measured the albumin concentration in cerebrospinal fluid, a condition that has been thought to depend on the caveolae-mediated transport of albumin from the blood, they found it surprisingly to be normal. It is possible that other undetermined defects in the endothelium of Cav-1-deficient mice (e.g., more porous paracellular routes) allow for a small but sufficient steady-state shunting of molecules.

5. Caveolin-1 in the Lung. The architecture of the alveolar septa (the primary site of gas exchange in lungs) is a thin cytoplasmic extension of the type I pneumocyte, a negligible layer of basement membrane/interstitial matrix, and the thin-walled endothelial cell. Despite this dearth of tissue mass (~0.6–1 μm in thickness), up to 70% of the total plasma membrane in a typical septa is estimated to be composed of caveolae (Gumbleton, 2001). This is mostly due to the fact that both endothelial cells and type I pneumocytes express Cav-1 and Cav-2 at high levels; thus caveolae, in both plasmalemmal and fully invaginated forms crowd the cytoplasmic extensions of both cell types (Dormans, 1983). By sheer magnitude, these structures and their marker proteins must serve an important role in lung physiology; yet, at the present, researchers have very little insight into the possible functions (proposed roles include involvement in gas exchange, endocytosis, signal transduction, and/or responses to shear/mechanical stress) (Lisanti et al., 1994b; Schnitzer et al., 1995c, 1996; Park et al., 2000; Gumbleton 2001; Predescu et al., 2001). Unfortunately, none of the studies have shown convincing lung-specific functions for caveolae and the caveolins. In this respect, the finding of lung defects in Cav-1-deficient mice was extremely enlightening.

Loss of lung caveolae results in significant histological lung pathology: the diameter of the alveolar spaces is

reduced and the alveolar walls are thickened and hypercellular (Fig. 14) (Drab et al., 2001; Razani et al., 2001a). Immunohistochemical analysis using Ki67, a marker of cellular proliferation, shows markedly increased cell proliferation in Cav-1 null lungs (Razani et al., 2001a). Similar analysis with an endothelial marker, Flk-1 (vascular endothelial growth factor receptor), reveals an increase in endothelial cell staining in Cav-1 null mouse lung as well. Taken together, these data suggest an endothelial cell proliferation phenotype in the Cav-1 KO lung. Cell cycle derangement might be presumed to underlie this lung phenotype given the faster proliferation and increased S-phase of primary Cav-1 null MEFs. Why obvious hypercellularity is not observed in other Cav-1 null tissues remains to be examined, although there are several possible explanations. For example, caveolae may play a relatively specific role in lung tissue or modifying factors may exist in other tissues that ameliorate the effects due to Cav-1 loss. Alternatively, more indirect mechanisms may be present, such as a compensatory increase in cell number due to the ineffective function of the Cav-1 null lung parenchyma.

In addition to the hypercellularity, Cav-1-deficient lungs also have an increased deposition of extracellular matrix. However, the two reports of this finding conflict on the composition of the deposition. Drab et al. (2001) noted increased trichrome-staining (consistent with fibrosis/collagen deposition), whereas Razani et al. noted increased reticulin staining (consistent with thickened basement membranes) with negligible trichrome staining. This difference is likely secondary to sample interpretation and will be resolved pending other confirmatory histological stains.

Nevertheless, increases in cell number and extracellular matrix are commonly seen in the restrictive lung diseases (a group of etiologically diverse disorders where thickening of alveolar septa results in reduced gas exchange and lung compliance, in turn leading to ineffective pulmonary function and significant morbidity/mortality). Examples of disorders with this manifestation include acute respiratory distress syndrome, idiopathic pulmonary fibrosis, and pneumoconiosis. Consistent with a restrictive picture, Cav-1-deficient mice are easily fatigued and markedly exercise-intolerant in swimming assays of exercise capacity (Drab et al., 2001; Razani et al., 2001a). Of course, detailed insight into the phenotype of Cav-1 null lungs requires a more rigorous assessment of lung physiology, including pulmonary function testing, whole body plethysmography, measurements of arterial blood gases, etc.

Given the extraordinary abundance of caveolae in the lungs and the complete lack thereof in Cav-1 null mice, one can easily surmise that such drastic pathology is directly due to a loss of morphologically identifiable caveolae in the lungs. This attractive interpretation of these phenotypes was complicated recently when we reported the identical pathology in mice deficient in caveolin-2, the other caveolin gene found highly expressed in all Cav-1-expressing tissues (Fig. 14) (Razani et al., 2002b) (see *Section VI.B.*).

6. The Vascular Physiology of Caveolin-1-Deficient Mice. As mentioned above, the caveolin-1/eNOS signaling connection has been well studied in the past few years with much evidence supporting its physiological significance. Recent studies on the vasoresponsiveness of isolated aortic rings from Cav-1-deficient mice have now bolstered this connection. Cav-1 null aortas have a blunted response to phenylephrine-induced vasoconstriction and are hyper-responsive to Ach-induced vasorelaxation (Fig. 13B) (Drab et al., 2001; Razani et al., 2001a). Furthermore, the vasodilatory tendencies of Cav-1 null aortas can be completely abrogated by the addition of L-NAME, a potent eNOS inhibitor (Razani et al., 2001a). Taken together, these data suggest that in the absence of caveolin-1, eNOS not only retains a higher level of activity at baseline but lacks the regulatory component by which it can be turned off after the arrival of stimuli such as Ach (a mechanism akin to that proposed in Fig. 9). In direct support of constitutive eNOS activation, Cav-1 null vascular smooth muscle cells also have a severalfold increase in NO and its downstream mediator, cGMP (Drab et al., 2001).

Perturbations in NO and NOS activity have been implicated in numerous disease processes ranging from

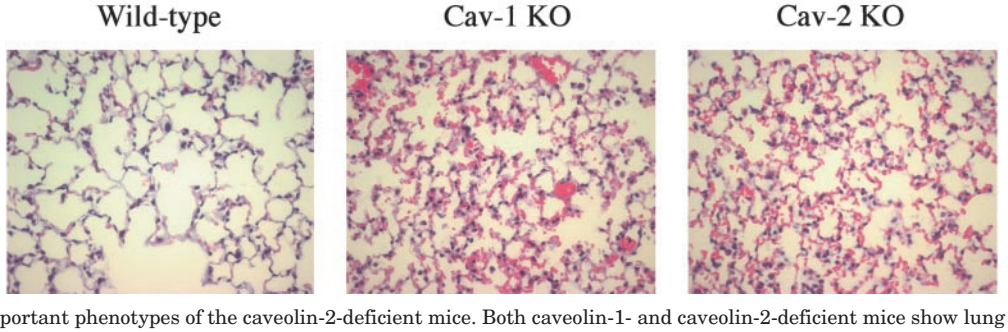

FIG 14. Some important phenotypes of the caveolin-2-deficient mice. Both caveolin-1- and caveolin-2-deficient mice show lung abnormalities, with constricted alveolar spaces, thickened septa, and hypercellularity. Shown is H&E staining of lung tissue from wild-type, Cav-1 null (Cav-1 KO), and Cav-2 null (Cav-2 KO) mice (Razani et al., 2001a, 2002b). As tissues in Cav-1 null mice also lack Cav-2 expression due to protein destabilization, the identical lung phenotype observed in both Cav-1- and Cav-2-deficient mice attests to the importance of caveolin-2 in the proper functioning of caveolae in the lung.

hypertension, responses to shock, and inflammation. As described above, Cav-1 clearly serves a "modulatory" role in eNOS function.

7. Caveolin-1 and Lipid Homeostasis. Despite a strong connection between caveolae, caveolin-1, and adipose function/lipid storage, initial analysis of Cav-1 null mice showed no obvious adipocyte pathology, with intraperitoneal fat pads remaining histologically normal (Drab et al., 2001; Razani et al., 2001a). However, if Cav-1 null mice are allowed to grow older, an interesting observation is made: they have significantly smaller body sizes than their wild-type counterparts. With some indication that this phenotype might be related to alterations in adipose tissue, Razani et al. (2002a) conducted a rigorous metabolic analysis of Cav-1-deficient mice.

Despite being hyperphagic, Cav-1 null mice show an overt resistance to diet-induced obesity as they grow older (Fig. 13C). These mice are lean, have dramatically smaller fat pads, histologically reduced adipocyte cell diameters, and a poorly differentiated/hypercellular white adipose parenchyma (Fig. 13C). Although such dramatic adipose pathology is not observed in young mice, a detailed histological analysis of the fat pads in young Cav-1 null mice shows that mammary gland adipocytes are highly atrophic and the hypodermal fat layer in both male and female mice is completely ablated. Therefore, it appears that the lipodystrophic changes in Cav-1 null adipocytes initially affect certain sites more than others, but become pervasive as the mice age.

Furthermore, Cav-1 null mice are highly deranged metabolically, even at a young age. They have severely elevated triglyceride and free fatty acid levels, especially in the postprandial state. Lipoprotein profile analysis showed that there is a selective build-up of chylomicrons/VLDLs in these mice, and kinetic studies indicated that this build-up is most likely due to poor clearance of postprandial lipids by the adipocytes.

At this juncture, the mechanism of the elevation in triglycerides/free fatty acids is not known. As a deficiency in lipoprotein lipase is considered the major culprit in isolated hypertriglyceridemia, Razani et al. (2001a) assessed its in situ enzymatic activity in Cav-1 null mice but found it to be normal. They also noted that irrespective of the lipid perturbations, insulin and glucose levels remain normal in these mice, suggesting that overt insulin resistance might not play a role in the lipid abnormalities. However, they did not go beyond this first-pass analysis, leaving several questions unanswered. Certainly, in light of the previously discussed roles for Cav-1 in lipid homeostasis (i.e., its ability to bind fatty acids, its targeting to lipid droplets) or its role in signal transduction including the insulin receptor signaling pathway (Yamamoto et al., 1998; Nystrom et al., 1999), it is plausible that some or all of these pathways are perturbed in caveolae-deficient adipocytes. Importantly, it should be noted that Cav-2-deficient mice, which do not show any effects on caveolae formation, have none of the adipocyte or lipid abnormalities of the Cav-1 null mice (Razani et al., 2001a; unpublished observations), indicating that Cav-1 and caveolae play a selective role in the entire range of metabolic phenotypes observed here.

B. Studies of Caveolin-2-Deficient Mice

The near complete loss of Cav-2 protein levels in Cav-1 null tissues leaves unresolved whether any of the phenotypes observed in the Cav-1 null setting are related to the reduction of Cav-2. To approach this question directly, we generated Cav-2-deficient mice (Razani et al., 2002b). These mice are viable, fertile, and demonstrate no obvious abnormalities upon general inspection. Interestingly, electron microscopic analysis reveals that caveolae remain generally unaffected by the loss of Cav-2, maintaining a morphology and cellular distribution seen in wild-type animals. The generation of Cav-2 null mice thus allows for the analysis of two issues: 1) identifying the specific role of Cav-2; and 2) examining the functionality of caveolae formed purely by Cav-1. As detailed below, the phenotypes observed in Cav-2 null mice were anything but predictable (for a summary, see Table 4).

1. Relationship with Caveolin-1. In the absence of Cav-2, Cav-1 protein expression and membrane localization is in large part intact (Razani et al., 2002b). Caveolin-1 is able to homo-oligomerize, traffic to the plasma membrane, and form caveolae. However, Cav-1 expression is decreased by as much as 2-fold in certain tissues (e.g., the lung), yet unaffected in others (e.g., adipose tissue). Although this is not a dramatic alteration in expression, it likely signifies slight instabilities in the caveolin oligomeric complex, albeit not enough to disrupt caveolin homo-oligomerization or the structural integrity of caveolae in vivo.

2. The Surprising Role of Caveolin-2 in the Lung. Initial characterization of Cav-2 null mice revealed that the absence of Cav-2 results in defects in only one major organ system, the lungs. Despite an intact caveolae membrane system, the Cav-2 null lung parenchyma shows hypercellularity with thickened alveolar septa and an increase in the number of endothelial cells, a histological picture that is virtually indistinguishable from that of the Cav-1 null mice (Fig. 14). In addition, in the Cav-2-deficient setting, these mice are markedly exercise-intolerant, to the same levels observed for the Cav-1 null mice. As mentioned before, Cav-1 expression is diminished by ~2-fold in these lungs; however, the observed pathology cannot be due to this slight perturbation since Cav-1 heterozygous animals (who have a similar decrease in Cav-1 expression) fail to exhibit any lung pathology (Razani et al., 2002b). Thus, it appears

that the morphologic loss of caveolae is not required for proper lung function; rather, it is perturbations in the caveolar coat proteins that play a more significant role. This result is extremely important for two reasons: 1) it shows that the phenotypes present in the Cav-1 knockout are not all directly due to a loss of caveolae or the sole function of Cav-1; and 2) it is the first time a specific role for caveolin-2, independent of Cav-1, has ever been proposed.

Importantly, the other prominent phenotypes observed in Cav-1 null mice (i.e., altered vasoconstriction/relaxation responses, abnormal diet-induced obesity and lipid profiles) are not observed in the Cav-2 mice (Razani et al., 2002b). This suggests that Cav-1/Cav-2 hetero-oligomers play an important and distinct role from Cav-1 homo-oligomers in lung tissue.

C. Studies of Caveolin-3-Deficient Mice

To gain a better understanding of the role played by caveolin-3 in a whole animal system, we and others have recently generated caveolin-3-deficient mice using standard homologous recombination techniques (Hagiwara et al., 2000; Galbiati et al., 2001a). These mice are viable, fertile, and demonstrate no obvious abnormalities upon general inspection. Electron microscopic analysis reveals a loss of caveolae in all striated muscle cells, thus definitively demonstrating the necessity of Cav-3 for caveolae formation in striated muscle (Fig. 15A) (Hagiwara et al., 2000; Galbiati et al., 2001a; and unpublished data). Table 4 provides a summary of the features of Cav-3 null mice that offers insights into the roles of caveolae in these and other cell types.

1. Caveolin-3 and Muscle Disease. Mutations in Cav-3 that cause muscle disease were first reported by Minetti et al. (1998) who identified two families, each with different autosomal dominant mutations, that result in LGMD-1C. Additional cases of LGMD-1C have been reported since, making a total of five distinct Cav-3 mutations to date that are causal for LGMD-1C (Herrmann et al., 2000; Matsuda et al., 2001). Interestingly, mutations in Cav-3 have also been associated with three other muscle diseases: rippling muscle disease, distal myopathy, and idiopathic elevated creatine kinase levels (hyperCKemia) (Carbone et al., 2000; Betz et al., 2001; Vorgerd et al., 2001; Tateyama et al., 2002). It is thus clear that the genetic background in which mutant Cav-3 is expressed affects the ultimate phenotype as the same mutation in Cav-3 can result in distinct muscle diseases. Common to all these diseases, however, is a reduction or near elimination of Cav-3 expression. Mice deficient for caveolin-3 thus represent an animal model for diseases in which caveolin-3 is markedly reduced. Histological analysis of skeletal muscle tissue from caveolin-3 null mice reveals mild myopathic changes, with variability in the size of muscle fibers, necrotic fibers, and a mononuclear cell infiltration, consistent with the features of LGMD-1C pathology (Fig. 15B) (Hagiwara et al., 2000; Galbiati et al., 2001a).

Interestingly, Cav-3 expression is elevated in Duchenne muscular dystrophy and its mouse model, mdx (Bonilla et al., 1981; Vaghy et al., 1998). This indicates that proper control of Cav-3 expression may be important for normal muscle homeostasis. In support of this conclusion, transgenic mice overexpressing mutant or wild-type Cav-3 have severely affected skeletal muscle both histologically and functionally (Sunada et al., 2001).

2. Caveolin-3 and Transverse Tubules. The coalescence of caveolae to form the transverse tubule (T-tubule) system in muscle was first indicated by EM studies that predated the identification of the Cav-3 gene (Schiaffino et al., 1977). Later, Cav-3 was shown to be transiently associated with the developing T-tubule system using immunogold labeling with Cav-3 antibodies (Parton et al., 1997). The generation of Cav-3-deficient mice provided the ideal system to investigate the necessity of Cav-3 in T-tubule development/maintenance. Immunostaining of mature Cav-3 null skeletal muscle with antibodies to the dihydropyridine receptor-1α and ryanodine receptor, two well characterized T-tubule markers, showed diffuse and disorganized labeling, suggestive of structural abnormalities in the T-tubule system (Galbiati et al., 2001a). EM analysis of ferrocyanate-stained mature Cav-3 KO skeletal muscle showed dilated and disorganized/longitudinally oriented T-tubules (Fig. 15C). Longitudinally oriented T-tubules are normally seen only in developing skeletal muscle. Interestingly, dilated and developmentally abnormal T-tubules have recently been observed in patients with LGMD-1C (Minetti et al., 2001). Thus, it appears that the proper development and function of the skeletal T-tubule system requires caveolin-3. The detailed molecular underpinnings of the T-tubule abnormality and its relevance to other caveolin-3-deficient pathologies awaits further investigation.

3. Caveolin-3 and the Dystrophin-Glycoprotein Complex. Several components of the DG complex are concentrated in caveolae (Doyle et al., 2000; Galbiati et al., 2001a). Although Cav-3 is not an integral member of the DG complex (Crosbie et al., 1998), Cav-3 can associate with the complex through a novel WW domain (Sotgia et al., 2000). Examination of DG complex members in Cav-3-deficient animals reveals no alteration in their expression or membrane association; however, biochemical fractionation of the muscle cell plasma membrane reveals dystrophin and several of its associated proteins to be excluded from lipid raft microdomains/caveolae (Galbiati et al., 2001a). This indicates that caveolar localization of at least a portion of the DG complex is critical for the maintenance of skeletal muscle structural integrity. In future studies, it will be important to further dissect

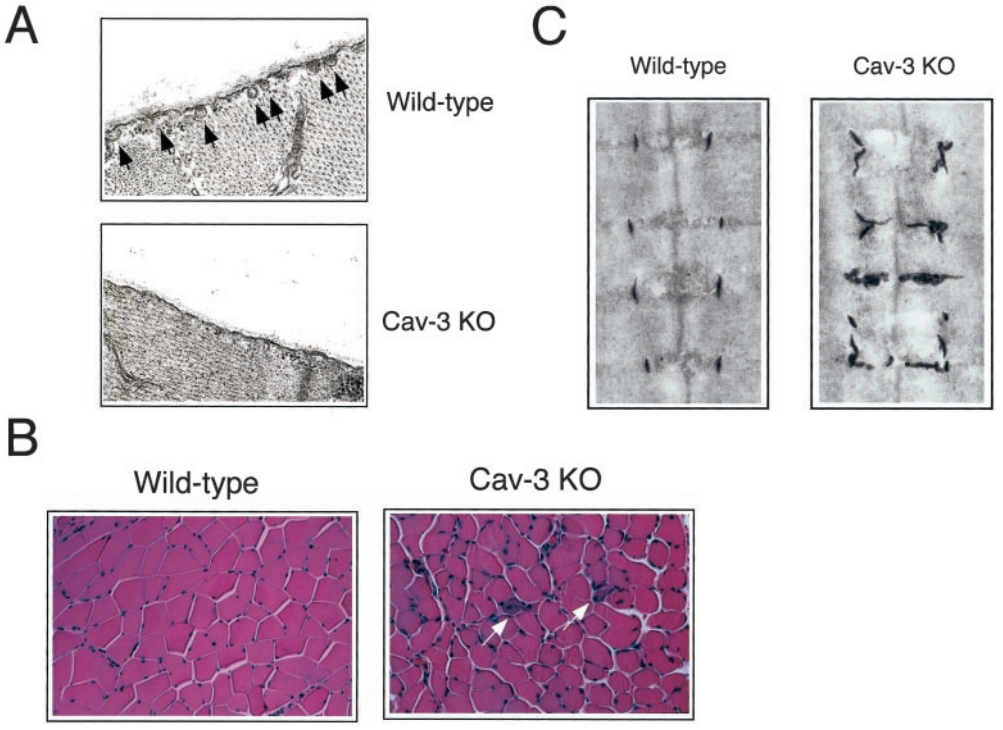

FIG 15. Some important phenotypes of caveolin-3-deficient mice. A, a deficiency in Cav-3 is sufficient to completely disrupt formation of sarcolemmal caveolae. Tissues thus far examined include skeletal muscle from various locations. The transmission electron micrographs shown are from hindlimb skeletal muscle fibers imaged at 25,000× magnification (Galbiati et al., 2001a). B, caveolin-3-deficient mice show skeletal muscle abnormalities, with variability in muscle fiber size, presence of some necrotic fibers, and mononuclear infiltrates. Muscle tissue sections from wild-type (left panel) and Cav-3 KO mice (right panel) were cut and stained with H&E. C, caveolin-3 protein expression is required for the development of a mature and highly organized T-tubule system. Cav-3 KO T-tubules are dilated and run in irregular directions. Skeletal muscle tissue samples from wild-type (left panel) and Cav-3 KO (right panel) were subjected to T-tubule system staining using potassium ferrocyanate, which gives T-tubules an electron-dense (black) appearance (Galbiati et al., 2001a).

the interactions between components of the dystrophin complex in Cav-3 null mice.

D. Dominant-Negative Caveolin Mutations in Human Disease

There are 12 amino acid residues that are conserved within all caveolins from worms to humans (Fig. 16). Each of the muscle diseases associated with Cav-3 involves mutations in one of these residues. The first to be discovered and best characterized are a Pro → Leu substitution at amino acid position 104 (P104L) and a deletion of 3 amino acids at positions 63–65 (ΔTFT) that result in LGMD-1C (Minetti et al., 1998). These LGMD-1C patients demonstrated a ~95% reduction in Cav-3 levels. The P104L and ΔTFT mutant Cav-3 proteins act in a dominant-negative fashion by hetero-oligomerizing with wild-type Cav-3 and directing their proteasomal degradation (Galbiati et al., 2000b). Two additional mutations in Cav-3 that result in LGMD-1C have been identified: A45T and T64P (Herrmann et al., 2000). Each of these mutations also results in a marked reduction in Cav-3 expression in skeletal muscle. Although a detailed molecular characterization of these mutants has not been performed, given their autosomal dominant inheritance, they too most likely act to sequester wild-type Cav-3 in a dominant-negative fashion.

It is intriguing that some of the same mutations that result in LGMD-1C have been shown to be associated with other muscle disorders. Multiple families with autosomal dominant rippling muscle disease (RMD) harbor Cav-3 mutations: R26Q, A45T, A45V, and P104L (Betz et al., 2001). A sporadic case of RMD due to a R26Q Cav-3 mutation has also been documented (Vorgerd et al., 2001). Recently, an R26Q Cav-3 mutation due to a heterozygous nucleotide substitution was identified in a patient with a distal myopathy (Tateyama et al., 2002). In both the RMD and distal myopathy cases, a near elimination of Cav-3 expression is seen. Finally, a sporadic R26Q mutation in Cav-3 was demonstrated in two patients with elevated creatine kinase levels (hyperCKemia) (Carbone et al., 2000). Although a ~65% decrease in Cav-3 expression was shown in these patients, they did not manifest the paradigm symptoms of LGMD-1C, RMD, or distal myopathy. Disease-causing mutations in Cav-3 are in residues that cause a dominant-negative phenotype, one in which the Cav-3 mutant disrupts the normal functioning of the wild-type Cav-3 protein. How what appear to be a variety of dominant-negative mutations in Cav-3 can manifest as distinctly different muscle dysfunctions awaits further analysis.

Given the conservation across species of certain amino acid residues in caveolins and the proven disease-caus-

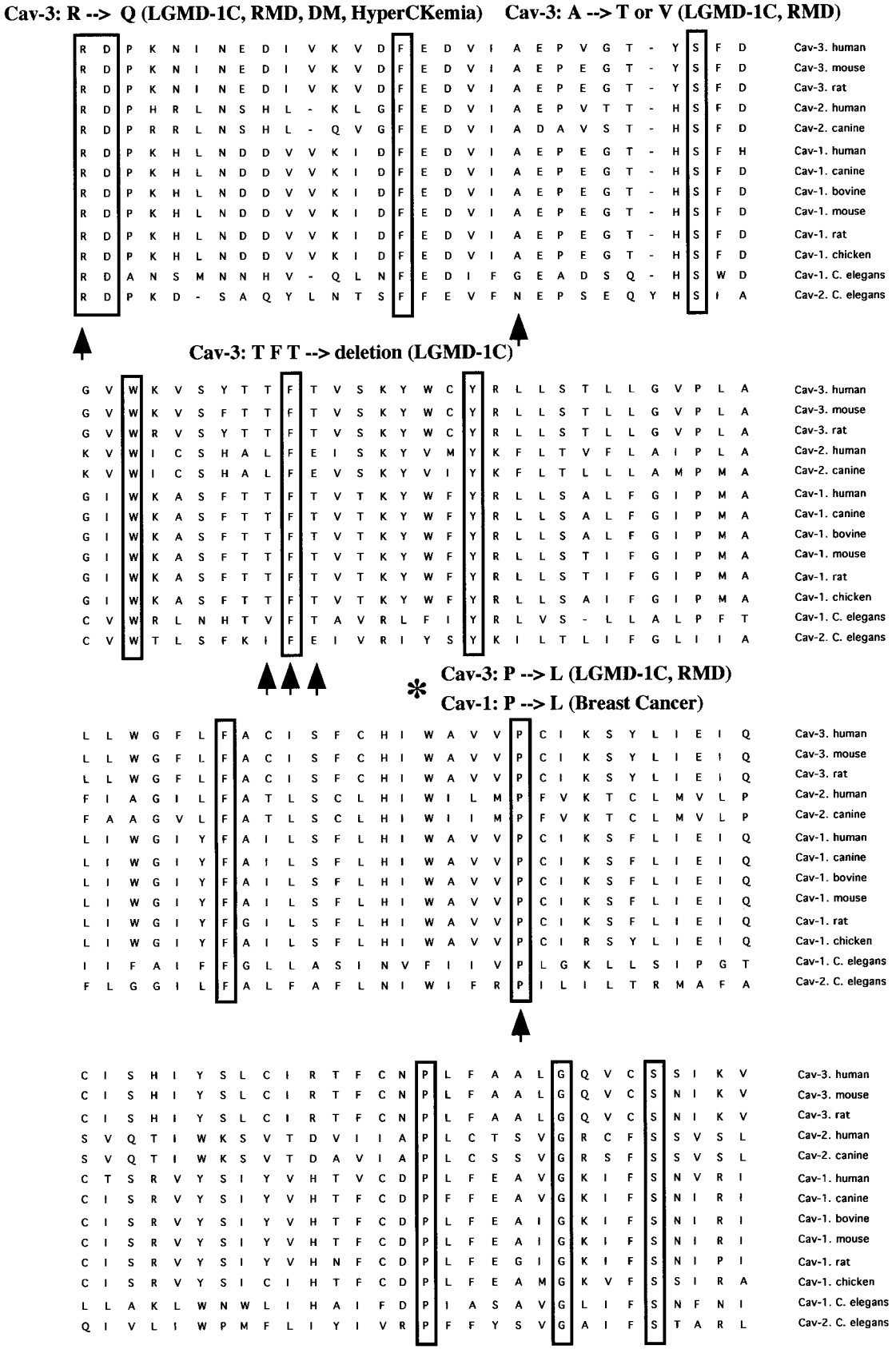

FIG 16. Dominant-negative disease-causing mutations in human Cav-1 and Cav-3. Sequence alignment of all the known caveolins indicates that most of the disease mutations found in human caveolin-3 (R26Q, ΔTFT, and P104L) and human caveolin-1 (P132L) coincide with one of the 12 residues (demarcated by boxes) that are conserved in all caveolin genes, from *C. elegans* to humans. Interestingly, many of these mutations seem to act in dominant-negative fashion by mis-localizing or causing the degradation of the wild-type form of the caveolin proteins. RMD, rippling muscle disease; DM, distal myopathy.

ing mutations in these residues in Cav-3, it might be predicted that mutations in the corresponding residues of Cav-1 and -2 would be deleterious. In point of fact, it was recently reported that the Cav-1 gene is mutated in up to 16% of human breast cancer samples examined. The mutation results in a Pro → Leu amino acid substitution, Cav-1 (P132L). This mutation is analogous to the Cav-3 (P104L) mutation (Fig. 16) (Hayashi et al., 2001). Recombinant expression of the Cav-1 P132L mutant in NIH 3T3 cells was sufficient to cause cellular transformation (Hayashi et al., 2001). Anti-sense ablation of Cav-1 expression results in a similarly transformed cellular phenotype (Galbiati et al., 1998). It is most likely that Cav-1 P132L acts in a dominant-negative fashion. This first description of a mutation in the Cav-1 gene is sure to be followed by the identification of many similar dominant-negative caveolin mutations in other human diseases.

VII. Conclusions and Future Directions

A. Caveolae and Caveolins

Scientific research in a particular area is often marked by pivotal moments. The ultrastructural identification of caveolae in the 1950s and the cloning of each of the caveolins in the 1990s are two such moments in the caveolin field. The next era of caveolin research has now just begun with the generation and individual characterization of caveolin knock-out mice. These mice mark the first in vivo functional assessment of caveolins/caveolae. The phenotypes of the Cav-deficient mice have been everything from i) unpredicted, ii) to supportive of previous hypotheses, iii) to cause for reformulating prior notions.

Given that with the loss of Cav-1 or Cav-3, the cell loses not only a protein but an entire membrane domain in multiple organ systems, it might have been posited that Cav deficiency would result in the lethality of the mice. However, it must be considered that most disease-associated genes are not essential for viability. As mutations in Cav-1 and Cav-3 have been associated with human diseases, their proper expression is obviously not necessary for viability.

Despite the intricate role of Cav-1 in cholesterol trafficking and homeostasis and its proposed roles in atherosclerosis, Cav-1 null mice do not appear to have altered serum cholesterol levels (Drab et al., 2001; Razani et al., 2002a). Obviously, a more rigorous analysis needs to be conducted including lipid influx/efflux experiments, placement of Cav-1 null cohorts on atherogenic diets, and double-crosses with hypercholesterolemic mouse models.

Despite the down-regulation of Cav-1 in numerous tumors and its ability to regulate several oncogenic pathways (both suggestive of a tumor-suppressive role), Cav-1 null mice do not present with a higher incidence of carcinomas (Razani et al., 2001a). However, they do manifest hyperproliferative characteristics both in vitro and in vivo (Drab et al., 2001; Razani et al., 2001a), indicating that full onset tumorigenesis might require the synergistic targeted disruption of Cav-1 and other classically known tumor suppressors. In this regard, treatment of these mice with carcinogens and/or crosses with tumor-prone mice will be an important next step.

Upon identifying multiple signaling molecules within the caveolar fractions of cells, Lisanti and colleagues (1994a) proposed the "caveolae signaling hypothesis". This hypothesis states that the compartmentalization of signaling molecules allows for the regulation of signal transduction by bringing interacting molecules into proximity with one another and/or by sequestering molecules away from the rest of the cellular pool. Through multiple in vitro experiments, eNOS has perhaps been the best characterized of the caveolar localized molecules. The exaggerated vasorelaxative response in the Cav-1 null mice is a clear indication of the hyperactivation of eNOS due to loss of Cav-1 inhibition (Drab et al., 2001; Razani et al., 2001a). The analysis of other putative Cav-1 inhibited molecules awaits further investigation (Smart et al., 1999).

Another fruitful area of future research is the characterization of mice with multiple caveolins knocked out. Cav-1 null mice still have caveolae in all their striated muscle cells and Cav-3 mice have caveolae remaining in their nonmuscle cells. Thus a truly "caveolae-less" mouse model has not been characterized. Whole organism ablation of caveolae will only come about by intercrossing Cav-1 KO and Cav-3 KO mice to yield a Cav-1/3 double knock-out mice. It will be especially interesting to examine tissues that abundantly express both Cav-1 and Cav-3, such as the heart, in Cav-1/3 double knock-out animals. The smooth muscle cell, which is the only known cell to express both Cav-1 and Cav-3 will also be of major interest. In addition, Cav-2/3 and Cav-1/2/3 knock-out crosses may illicit as yet unimagined phenotypes, which will further reveal the functional roles of the caveolins/caveolae.

This new era of caveolar research (brought to the forefront by the generation of caveolin-deficient mice) is sure to yield greater insight into the physiological and pathological roles of the different caveolin family members.

B. Modifiers of Raft Function

Several different classes of lipid rafts are now believed to coexist in a single mammalian cell. Besides classical lipid rafts, which lack structural protein components, liquid-ordered domains may be enriched in one particular structural protein component, which drastically changes the morphology and/or the function of the lipid raft. Here, we refer to this newly emerging class of structural proteins as MORFs (modifiers of raft function). The first MORF to be identified was the caveolin-1 protein. When caveolin-1 is integrated into the microen-

vironment of a lipid raft, these microdomains invaginate and form caveolae, i.e., 50- to 100-nm flask-shaped structures located at or near the plasma membrane.

Besides caveolins, several other protein families have been recently reported to structurally and functionally modify lipid rafts. These MORF proteins include the flotillins (FLO-1 and -2; a.k.a. reggies or cavatellins), LAT/PAG, MAL/BENE, stomatins, and VIP36 (Galbiati et al., 2001b). Each of these MORFs may be responsible for the formation of a distinct class of lipid raft. Future studies will have to address the detailed functions of this newly emerging class of *raft modifier* integral membrane proteins.

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