Published in final edited form as:

Traffic. 2013 June ; 14(6): 663–677. doi:10.1111/tra.12066.

Overexpression of caveolin-1 is sufficient to phenocopy the behavior of a disease-associated mutant

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Abstract

Mutations and alterations in caveolin-1 expression levels have been linked to a number of human diseases. How misregulation of caveolin-1 contributes to disease is not fully understood, but has been proposed to involve the intracellular accumulation of mutant forms of the protein. To better understand the molecular basis for trafficking defects that trap caveolin-1 intracellularly, we compared the properties of a GFP-tagged version of caveolin-1 P132L, a mutant form of caveolin-1 previously linked to breast cancer, with wild type caveolin-1. Unexpectedly, wild type caveolin-1-GFP also accumulated intracellularly, leading us to examine the mechanisms underlying the abnormal localization of the wild type and mutant protein in more detail. We show that both the nature of the tag and cellular context impact the subcellular distribution of caveolin-1, demonstrate that even the wild type form of caveolin-1 can function as a dominant negative under some conditions, and identify specific conformation changes associated with incorrectly targeted forms of the protein. In addition, we find intracellular caveolin-1 is phosphorylated on Tyr14, but phosphorylation is not required for mistrafficking of the protein. These findings identify novel properties of mistargeted forms of caveolin-1 and raise the possibility that common trafficking defects underlie diseases associated with overexpression and mutations in caveolin-1.

Keywords

caveolin; caveolae; fluorescence microscopy; breast cancer; GFP; dominant negative

INTRODUCTION

Caveolae are flask-shaped invaginations of the plasma membrane that function in a variety of cellular processes including intracellular trafficking, cell signaling, cholesterol

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homeostasis, and mechanotransduction (1, 2). In addition to gangliosides and cholesterol, caveolae require the 21 kDa protein caveolin-1 as well as proteins in the newly described cavin family to form (3-8). Caveolae biogenesis is initiated by the association of newly synthesized caveolin-1 with the endoplasmic reticulum, followed by its transport to the Golgi complex where it undergoes oligomerization and becomes associated with cholesterol, forming detergent-insoluble complexes (9-12). These complexes are then packaged into vesicles that are targeted to and insert into the plasma membrane, where they later incorporate cavins (12, 13). Once formed, caveolae are stable structures, and can be triggered to undergo endocytosis as well as undergo dynamic motions (14-17). When the assembly of caveolae is compromised by cholesterol depletion, overexpression of caveolin-1, or the loss of cavin, caveolin-1 forms small oligomers at the plasma membrane that are internalized and targeted for degradation in lysosomes (18).

Caveolin-1 is part of a larger gene family consisting of three family members, including caveolin-2 and the muscle-specific isoform caveolin-3 (1, 2). Caveolin-1 associates with cell membranes via a putative 33-amino acid domain that inserts into the cytoplasmic face of membranes, forming a hairpin-like structure with cytoplasmically oriented N- and C-termini (11). Two caveolin-1 isoforms, caveolin-1 α and 1 β , have been identified, with caveolin-1 α containing 32 N-terminal residues not present in caveolin-1 β (19). Caveolin-1 α is a substrate for phosphorylation by Src on Tyr14 (20), an event that has been linked to regulation of caveolae trafficking (21).

Both alterations in caveolin expression levels and mutations in the caveolins have been associated with a number of human diseases (22-28). For example, a P132L mutation of caveolin-1 has been associated with breast cancer (29-32), while a mutation in an equivalent residue in caveolin-3 is associated with muscular dystrophy (33). A common feature of several disease-associated caveolin mutants is that they accumulate intracellularly in the perinuclear region rather than targeting correctly to caveolae (30, 34-37). They also often exhibit defects in oligomerization state (30, 36) and are rapidly turned over (35). Several of these mutants have also been reported to have a dominant negative phenotype, as defined by their ability to cause wild type forms of caveolin to accumulate intracellularly as well upon coexpression (30, 35-38). The mechanism responsible for the mistrafficking of caveolin under these conditions is not fully understood, but has been hypothesized to involve conformational defects that prevent the protein from exiting the Golgi complex efficiently (39). For the case of the caveolin-1 P132L mutant, expression of the mutant protein has been reported to have both loss of function and dominant negative activities (32). Recent structural work on purified caveolin has now begun to identify the impact of the P132L mutation on the oligomerization state and structure of the protein (40). How these defects in caveolin structure and function give rise to human disease is not yet entirely clear, but has been speculated to be linked to the mislocalization of the protein within the cell (32).

In the current study, we sought to examine mechanisms that contribute to the intracellular accumulation of a dominant negative mutant of caveolin-1 associated with breast cancer, P132L. Unexpectedly, we found that overexpression of wild type caveolin-1-GFP (Cav1-GFP) is sufficient to give rise to a phenotype that mimics the behavior of P132L. These findings raise the possibility that intracellular accumulation of caveolin-1 represents a common mechanism underlying disease states associated with both overexpression and mutations of caveolin-1.

RESULTS

EGFP-tagged forms of both the breast cancer caveolin-1 mutant P132L and wt caveolin-1, but not caveolin-1-mCherry, accumulate in a perinuclear compartment in COS-7 cells

To investigate mechanisms underlying the intracellular accumulation of disease-associated forms of caveolin-1, we took advantage of a previously reported assay in which caveolin mutants and wild type forms of caveolin are tagged with either GFP or epitope tagged and then coexpressed (30, 35-37). To generate constructs to carry out these studies, we first used site directed mutagenesis to introduce the breast-cancer associated P132L mutation into a previously reported caveolin-1-EGFP (Cav1-GFP) construct (41), generating P132L Cav1-GFP. We also generated equivalent caveolin-1-mCherry (Cav1-mCherry and P132L mCherry) constructs for co-expression studies.

As a control, we first expressed each protein individually in COS-7 cells and examined their subcellular distribution by fluorescence microscopy. Based on previous findings, we predicted that P132L Cav1-GFP and P132L Cav1-mCherry would be trapped in a perinuclear compartment (30). In contrast, Cav1-GFP and Cav1-mCherry should either become incorporated into caveolae (30) or accumulate on the cell surface as small oligomers that are then trafficked to endolysosomes for degradation (18).

In agreement with previous findings (30), we found that P132L Cav1-GFP primarily accumulated intracellularly (Figure 1A). Unexpectedly however, Cav1-GFP had a similar distribution, with the dominant fluorescence signal emanating from the perinuclear region in the majority of cells (Figure 1B,C). Furthermore, the subcellular distribution of Cav1-mCherry and P132L-Cav1-mCherry were similar to one another (Figure 1 D-F), and were also markedly different than that of Cav1-GFP and P132L-Cav1-GFP. Two phenotypes were observed. In a subset of cells (20%, N = 68 cells), Cav1-mCherry was diffusely distributed on the plasma membrane and in an underlying reticular pattern characteristic of the endoplasmic reticulum (Figure 1E). In contrast, the majority of cells (80%, N = 68 cells) contained a perinuclear pool of Cav1-mCherry and bright fluorescent puncta within the cytoplasm in addition to diffuse plasma membrane staining (Figure 1F). The latter phenotype is reminiscent of the localization of Cav1-mCherry under conditions where the protein fails to incorporate into caveolae correctly (18). These findings suggest that the nature of the tag on caveolin-1 can have a significant effect on the subcellular distribution of the protein when it is transiently overexpressed, and that the P132L mutation is neither necessary nor sufficient to cause caveolin-1 to accumulate intracellularly.

Given the unexpected similarities in the subcellular localization of Cav1-GFP and Cav1-P132L and differences in localization of Cav1-mCherry and Cav1-GFP, we next sought to understand mechanisms that contribute to the perinuclear accumulation of the protein.

The severity of the perinuclear accumulation phenotype of caveolin-1 is dependent on the nature of the tag and cellular context

We first asked whether the differences in the subcellular localization of Cav1-GFP and Cav1-mCherry were the result of differences in the levels of overexpression of the two constructs. The expression levels were similar within error as assessed by western blotting (Supplementary Figure 1), ruling out this possibility. We additionally tested if the mistargeting of Cav1-GFP was a function of the degree of overexpression by decreasing the amount of plasmid DNA used to transfect cells (Figure 1 J-L). As the amount of plasmid DNA was decreased, an increasing fraction of the cells exhibited a phenotype in which Cav1-GFP was enriched in a few large punctate structures scattered throughout the cell (Figure 1 J-L). Thus, the perinuclear accumulation of Cav1-GFP occurs in a concentration-

dependent fashion. However, even at the lowest expression levels examined (Figure 1 J,K), the distribution of Cav1-GFP was distinct from that of Cav1-mCherry (Figure 1 E,F).

We next considered the possibility that the propensity of EGFP to weakly dimerize when attached to membrane proteins may contribute to its accumulation in the perinuclear region (42, 43). To test this, we introduced the A206K mutation (42) into the EGFP of the Cav1-GFP construct to generate caveolin-1-mGFP. Caveolin-1-mGFP accumulated intracellularly, similar to Cav1-GFP (Figure 1G), indicating that EGFP-EGFP interactions do not significantly contribute to the intracellular accumulation of Cav1-GFP.

The linker used to generate fusion proteins can potentially influence their trafficking and function (44). The P132L Cav1-GFP and Cav1-mGFP constructs described above were all generated from the same parent construct based on canine caveolin-1, Cav1-GFP (41), and thus also share the same linker. To test whether the linker may also contribute to the disrupted trafficking of these proteins, we constructed a novel Cav1 fusion utilizing the human caveolin-1 gene and a completely different linker, and coupled it to another GFP variant, mEmerald (Table 1). This construct also showed a predominantly perinuclear localization when expressed in COS-7 cells, similar to that observed for Cav1-GFP and P132L Cav1-GFP (Figure 1H). This result suggests the nature of linker does not account for the mistrafficking of the protein under these conditions.

The finding that Cav1-GFP and Cav1-mCherry have distinct subcellular localization patterns implies that the choice of tag attached to caveolin-1 can strongly influence the apparent localization and trafficking of the protein. To test this idea further, we examined the subcellular distribution of a previously described caveolin-1 construct that contained a small epitope tag, caveolin-1-myc (Cav1-myc) (45). Similar to the localization pattern typically observed for Cav1-mCherry, two phenotypes were observed for cells expressing Cav1-myc (Figure 1I). In one, Cav1-myc was primarily diffusely distributed in the plasma membrane/within a reticular pattern characteristic of the endoplasmic reticulum (37% of cells, N = 63) (Figure 1I, arrowheads), and in the second the protein was localized to a perinuclear compartment and a small number of punctate structures in addition to diffuse staining of plasma membrane (63% of cells, N = 63) (Figure 1I, arrows). This distinct difference in localization between Cav1-GFP and Cav1-myc further suggests the fate of exogenous caveolin-1 is strongly dependent on the nature of the tag as well as overexpression.

We wondered whether the distinct perinuclear accumulation of Cav1-GFP might also be cell-type specific. To address this question, we examined the distribution of the constructs described above in HeLa cells, where the localization and trafficking of fluorescent protein-tagged forms of caveolin-1 and P132L caveolin-1 have also been previously studied (12). To evaluate the presence of intracellular pools of caveolin-1, we co-stained cells for giantin to mark the Golgi complex. In general, when expressed in HeLa cells a large fraction of all of the caveolin-1 fusion proteins were localized to small puncta, as expected for a caveolae-associated protein, although in many cases diffuse labeling of the plasma membrane was also observed (Figure 2). Some colocalization of the various caveolin-1 fusion proteins with giantin was observed. The extent of colocalization varied substantially between cells, and tended to be higher for Cav1-GFP and Cav1-P132L than Cav1-mCherry and Cav1-mEmerald (data not shown).

The expression level of endogenous caveolin-1 varies widely between cell lines. To determine whether this could account for the cell-type specific differences in Cav1-GFP trafficking, we compared the expression levels of endogenous caveolin-1 in COS-7 and HeLa cells and found that they are essentially identical (Supplementary Figure 1). Thus, it

seems unlikely that differences in endogenous caveolin-1 expression levels cause Cav1-GFP to traffic differently in HeLa and COS-7 cells.

To further test for a possible contribution of endogenous expression levels of caveolin-1 to the mistrafficking of Cav1-GFP, as well as to extend our studies to an additional cell line, we compared the behavior of Cav1-GFP, P132L-GFP, Cav1-mCherry, and P132L-mCherry in caveolin-1^{+/+} and caveolin-1^{-/-} MEFs. In both cell lines, a significant pool of Cav1-GFP and Cav1-P132L were again confined to a perinuclear compartment, while Cav1-mCherry and Cav1-P132L tended to be distributed in punctate structures (Figure 3). We also observed a few caveolin-1 positive tubules in caveolin-1^{-/-} MEFs, consistent with a previous report that re-expression of caveolin-1 in cells lacking caveolin-1 can result in the formation of tubules (46). Since the localization of a given fusion protein was very similar in the caveolin-1^{+/+} and caveolin-1^{-/-} cells, we conclude that the cellular levels of endogenous caveolin-1 are not the major determinant of the fate of ectopically expressed caveolin-1.

We next focused on understanding the properties of mislocalized caveolin-1. Because the intracellular accumulation of Cav1-GFP was especially pronounced in COS-7 cells, we used this as a model for subsequent studies.

Cav1-GFP functions as a dominant negative in COS-7 cells

A number of caveolin mutants including P132L function as dominant negatives, causing the accumulation of wild type caveolin in the perinuclear region when the mutant and wild type proteins are coexpressed (30, 35-38). Given the similarity in both the subcellular distribution of P132L and wt caveolin-1-GFP, we wondered whether Cav1-GFP could act as a dominant negative. To test this, we took advantage of the finding that caveolin-1-myc or Cav1-mCherry are normally not extensively accumulated in the perinuclear region when expressed individually (Figure 1), and asked whether co-expression of Cav1-GFP causes them to become trapped within this region of the cell. Cav1-GFP P132L was used as a positive control for these studies, and EGFP served as a negative control.

To carry out these experiments, cells were cotransfected with Cav1-GFP, P132L Cav1-GFP, or EGFP in combination with either Cav1-mCherry or caveolin-1-myc (Figure 4). The next day, they were fixed and either visualized directly, or stained using an anti-myc antibody before imaging. As expected, expression of EGFP had no effect on the subcellular distribution of either Cav1-mCherry or caveolin-1-myc (Figure 4). In contrast, coexpression of wt Cav1-GFP or P132L Cav1-GFP caused a substantial fraction of caveolin-1-myc to become retained within the GFP-positive perinuclear compartment. When quantified over a large number of cells, Cav1-GFP demonstrated a more severe phenotype than did the P132L mutant (Figure 4). Cav1-GFP coexpression with P132L Cav1-mCherry also led to the intracellular accumulation of P132L Cav1-mCherry (Supplementary Figure 1).

These results demonstrate that wild type Cav1-GFP itself can function as a dominant negative, similar to the previously reported behavior of P132L caveolin-1 and several other disease-associated caveolin mutants. In order to gain further insight into whether similar mechanisms underlie their mistrafficking, we next asked whether Cav1-GFP and P132L Cav1-GFP share other common properties.

Cav1-GFP and Cav1-GFP P132L are rapidly turned over

Several caveolin mutants that accumulate intracellularly exhibit shortened half-lives compared to endogenous caveolin (35, 36). To test whether this is also the case for Cav1-GFP and P132L Cav1-GFP, we first asked whether the perinuclear pool of Cav1-GFP can be chased out by cycloheximide (CHX) treatment, as would be expected if it corresponds to newly synthesized caveolin-1 (39, 47, 48). As a positive control, we also examined

endogenous caveolin-1 levels in the Golgi complex. Levels of endogenous golgin-97 in the Golgi complex were monitored as a negative control.

To carry out these experiments, cells were either left untreated or incubated in the presence of CHX prior to fixation. They were then either mounted directly for visualization or processed further by immunostaining for caveolin-1 or golgin-97 (Figure 5). As expected, the levels of Golgi-associated endogenous caveolin-1 were significantly decreased in response to CHX treatment (Figure 5A, B). They were not however completely chased out, suggesting that a portion of endogenous caveolin in this region of the cell is not newly synthesized. In contrast, Cav1-GFP and P132L Cav1-GFP were readily depleted from the perinuclear region by CHX treatment (Figure 5C,D). Following CHX treatment, only minimal levels of GFP fluorescence could be detected throughout the cells, suggesting that Cav1-GFP is degraded, rather than trafficked to the cell surface under these conditions. These data imply that Cav1-GFP and P132L Cav1-GFP are rapidly cleared from the cell, consistent with previous reports of a shortened half-life of caveolin mutants.

Intracellular Cav1-GFP exhibits conformational defects as reported by altered epitope accessibility

Mutant forms of caveolin, including P132L, often exhibit defects in oligomerization (30, 36, 39), and conformational defects have been proposed to inhibit the trafficking of caveolin-1 mutants that accumulate intracellularly (39). To directly probe for disruptions in the conformation of caveolin-1 when the protein is trapped intracellularly, we took advantage of the fact that endogenous caveolin-1 normally exists in distinct forms in the Golgi complex and within caveolae that are recognized by antibodies raised against different domains of the protein. In particular, antibodies directed against either the scaffolding domain or C-terminus of caveolin-1 specifically recognize incompletely oligomerized caveolin-1 in the Golgi complex (12, 48-50). In contrast, N-terminally directed antibodies typically detect caveolin-1 within caveolae (41, 48, 49). Finally, a few antibodies detect caveolin both in the Golgi complex and caveolae (48).

We assembled a panel of antibodies that recognize different domains of caveolin-1 to probe the epitope accessibility of intracellular caveolin-1 (Table 2). We first determined the extent to which each of these antibodies recognizes endogenous caveolin-1 in the Golgi complex versus caveolae (Figure 6A). For these experiments, cells were fixed in PFA and permeabilized in saponin in order to preserve staining of caveolin-1 in the Golgi complex (48). As expected, several of the antibodies recognized caveolin-1 within caveolae and the perinuclear region (N20 and pAb h1-97). Others primarily labeled caveolin-1 within the Golgi complex (C-term and 2297), and yet others stained caveolae only (2234). Of these antibodies, both 2297 and 2234 tended to only weakly label endogenous caveolin-1 in COS-7 cells.

Surprisingly, two of the caveolin-1 antibodies we tested (C060 and C20B) showed completely different labeling patterns than any of the other antibodies (Supplementary Figure 3). Staining by mAb C060 was diffuse and spread throughout the cytoplasm, while C20B labeling was predominantly enriched in the perinuclear region, but was specifically excluded from the Golgi complex. Labeling of the nuclear envelope by both C20B and C060 could also often be observed (arrow, Supplementary Figure 3A). Given these unexpected staining patterns, we further analyzed their specificity in caveolin-1^{+/+} and caveolin-1^{-/-} MEFs. Unlike the other caveolin-1 antibodies such as h1-97, mAb C060 and mAb C20B each labeled similar structures in caveolin-1^{+/+} and caveolin-1^{-/-} MEFs (Supplementary Figure 3B, C), indicating that they cross-react with a currently unknown protein or proteins. They thus were not used for further analysis.

Having established the specificity of the various caveolin-1 antibodies for endogenous caveolin-1 within caveolae versus the Golgi complex, we next asked if the perinuclear accumulation of dominant negative forms of caveolin-1 was correlated with alterations in the conformation of caveolin-1 as reported by changes in epitope accessibility. To test this, we labeled cells expressing Cav1-GFP or P132L Cav1-GFP with the antibody panel. As a control, we stained cells expressing GalT-GFP (Supplementary Figure 4A). Staining of caveolin-1 was identical in cells expressing GalT-GFP and in untransfected controls. In contrast, we observed marked differences in caveolin-1 antibody staining patterns in cells expressing either Cav1-GFP (Figure 6B) or P132L Cav1-GFP (Figure 6C) compared to the distribution of the same antibodies in untransfected cells (Figure 6A). For example, Golgi staining for mAb C-term, normally present in essentially all untransfected cells, was completely absent in cells expressing Cav1-GFP or P132L Cav1-GFP.

Several antibodies labeled compartments of cells expressing Cav1-GFP or P132L Cav1-GFP that were typically poorly labeled in untransfected cells. Both mAb 2234 and mAb 2297 strongly labeled cells expressing Cav1-GFP and P132L Cav1-GFP in the perinuclear region (Figure 6B,C). Furthermore, mAb 2297 and mAb 2234 labeling often extended throughout the cell in the presence of Cav1-GFP or P132L caveolin-1-GFP, often including labeling of the ER and nuclear envelope (Figure 6B, C). Staining of the ER and nuclear envelope in transfected cells by pAb h1-97 and N20 could also sometimes be observed (Figure 6B,C). This ER-localized caveolin-1 pool likely corresponds to a weakly fluorescent form of Cav1-GFP and P132L caveolin-1-GFP, as the presence of the fusion proteins could be detected in this compartment by immunofluorescence staining using an anti-GFP antibody (Supplementary Figure 4B).

These findings suggest that substantial changes in the conformation of caveolin-1 occur when the protein is retained intracellularly. Moreover, similar changes in epitope accessibility were detected in cells expressing either wt or P132L Cav1-GFP, implying that they share common defects in folding and/or aggregation state.

Perinuclear Cav1-GFP and P132L Cav1-GFP are phosphorylated on Tyr14

Caveolin-1 can be phosphorylated by Src at Tyr14 (20). Given the substantial changes in the epitope accessibility of caveolin-1 in cells expressing Cav1-GFP or P132L Cav1-GFP, we wondered whether changes in the phosphorylation state of caveolin-1 might occur under these conditions. To test this, we stained untransfected cells and cells expressing Cav1-GFP using a PTyr14 caveolin-1 antibody (Figure 7). Consistent with previous reports [(51) and references therein], we found that in untransfected COS-7 cells, the primary site of antibody staining by the PTyr14 antibody was focal adhesions (Figure 7A). Low levels of punctate staining could sometimes also be detected (Figure 7A). In cells expressing caveolin-1-GFP, strong perinuclear PTyr14 caveolin-1 labeling signal was apparent in addition to low levels of focal adhesions staining (Figure 7E). PTyr14 staining of the perinuclear compartment was also observed in cells expressing P132L Cav1-GFP (Figure 7F).

Previous studies have indicated the commercially available PTyr14 caveolin-1 antibody used in our initial experiments cross-reacts with phosphopaxillin at a site speculated to correspond to Tyr118 of paxillin (51). We confirmed that the PTyr14 antibody stained focal adhesions in caveolin-1^{-/-} cells, indicating that this antibody does not solely bind to caveolin-1 (Figure 7C). This raised the question of whether the PTyr14 staining corresponds to phosphorylated caveolin, or instead to phosphorylated paxillin. We addressed this question in several ways. First, in order to further verify that the PTyr14 antibody is capable of recognizing a protein other than paxillin, we used it to stain paxillin^{-/-} MEFs. Focal adhesion staining was still apparent (Figure 7D), demonstrating that phosphopaxillin is not the only antigen recognized by this antibody. Next, we asked whether perinuclear staining

by the PTyr14 caveolin-1 antibody in Cav1-GFP expressing cells was due to the presence of phosphorylated paxillin within this compartment. To test this, we compared the staining patterns for paxillin and phosphopaxillin in cells expressing Cav1-GFP. Diffuse paxillin staining was observed, in addition to labeling of focal adhesions both in cells expressing caveolin-1-GFP and surrounding untransfected cells, indicating that paxillin is not enriched in the Cav1-GFP compartment (Figure 7G). Labeling by the phosphopaxillin antibody was also primarily excluded from the perinuclear region of cells expressing caveolin-1-GFP, although in some cells weak labeling could be detected, suggesting it may show some crossreactivity for phosphocaveolin-1 (Figure 7H). Finally, we tested a second commercially available PTyr14 caveolin-1 antibody, and found that it too strongly labeled Cav1-GFP (Supplementary Figure 5A).

Taken together, these data suggest the perinuclear staining by the PTyr14 caveolin-1 antibody in cells expressing Cav1-GFP reflects the specific recognition of Tyr14 phosphorylated caveolin-1 per se rather than phosphopaxillin. In addition, they raise the possibility that phosphorylation of Tyr14 plays a role in regulating the intracellular accumulation of caveolin-1-GFP.

Tyr14 phosphorylation is not required for the dominant negative phenotype of Cav1-GFP

We next asked whether Tyr14 phosphorylation is required for accumulation of Cav1-GFP in the perinuclear region. To test this, we examined a mutant form of Cav1-GFP in which Tyr14 has been mutated to Phe (52). When expressed in COS-7 cells, the subcellular distribution of Y14F Cav1-GFP was very similar to that of Cav1-GFP and P132L Cav1-GFP, localizing primarily in a brightly staining perinuclear compartment (Figure 7I). Consistent with the absence of the Tyr14 phosphorylation site, the mutant protein failed to be recognized by the PTyr14 caveolin-1 antibody (Figure 7I). It also was not labeled by paxillin or phospho-paxillin antibodies, further suggesting that the PTyr14 caveolin antibody staining of Cav1-GFP was specific for phosphocaveolin and was not due to crossreactivity with phosphopaxillin (Supplementary Figure 5B, C).

These findings imply Tyr14 phosphorylation of caveolin-1 is a consequence of the intracellular accumulation of the protein, rather than responsible for triggering its aberrant localization. However, this does not preclude the possibility that tyrosine phosphorylation is important for other aspects of the phenotype. We therefore performed additional experiments to test whether the Y14F mutant had similar behavior as wt Cav1-GFP and the P132L mutant.

As for the case of wt and P132L caveolin-1-GFP, we found that Y14F Cav1-GFP functioned as a dominant negative against Cav1-myc and Cav1-mCherry (Figure 8A-D) and rapidly turned over (data not shown). In addition, several of the changes in caveolin-1 epitope accessibility seen in response to the expression of Cav1-GFP and P132L were also observed in cells expressing the Y14F mutant, including enhanced labeling by mAb 2297 in the perinuclear region and endoplasmic reticulum, and the loss of Golgi staining by mAb C-term (Figure 8E). However, unlike cells expressing wt Cav1-GFP or P132L (Figure 6), no staining by mAb 2234 was observed for Y14F Cav1-GFP expressing cells (Figure 8E). Thus, phosphorylation of caveolin-1 on Tyr14 may be required to stabilize the protein in a conformation that allows for efficient recognition of the N-terminus by mAb 2234.

DISCUSSION

Cav1-GFP has served as an important reporter of the trafficking and properties of caveolae, and is properly incorporated into caveolae when expressed at low levels and/or in stable cell lines (12, 14, 16, 17, 41). Previous studies have shown that overexpression of caveolin-1 can

disrupt the normal trafficking of the protein by causing its delivery to the cell surface as small oligomers that are internalized and targeted for degradation in lysosomes (18). Our current results reveal a second phenotype associated with caveolin-1 overexpression. In particular, we show that overexpression of Cav1-GFP is sufficient to give rise a trafficking defect similar to that previously identified for a breast cancer associated mutant of caveolin-1, P132L (30). Furthermore, we identify several novel properties of the protein under conditions where it accumulates intracellularly.

The addition of GFP to the N-terminus of caveolin-1 was previously reported to generate a form of the protein that disrupts endocytosis of SV40 virus, despite the fact that the N-terminal fusion protein has a similar subcellular distribution as Cav1-GFP (41). Here, we show that the exact nature of the fusion tag on the C-terminus of caveolin-1 can strongly influence the intracellular trafficking of the protein. In particular, we found that when transiently overexpressed in COS-7 or MEF cells, Cav1-GFP, but not Cav1-mCherry or Cav1-myc, strongly accumulated in a perinuclear compartment, mimicking the previously reported behavior of P132L caveolin-1. Moreover, the properties of wild type Cav1-GFP and P132L Cav1-GFP were similar as assessed by several criteria, including their dominant negative activities, epitope accessibility, and Tyr14 phosphorylation status. Our results suggest the effects of caveolin-1 overexpression also depend on the cellular context, as in HeLa cells the perinuclear accumulation phenotype was significantly less severe than in COS-7 or MEF cells. We speculate that differences in the expression of either other proteins that contribute to caveolae formation or chaperones that assist with the folding of caveolin could potentially contribute to the cell-type dependent nature of the phenotype. The dependence of caveolin-1's localization on both tagging strategy and cell type may explain why some previous studies found that tagged wild type caveolin-1 did not function as a dominant negative while a P132L mutant form did (30).

It is not yet entirely clear why EGFP- and mCherry-tagged versions of caveolin-1 traffic differently from one another, although we ruled out a number of reasons why this could be the case. The simplest interpretation of our findings is that in certain cellular contexts, C-terminal tagging of caveolin with GFP can interfere with proper folding of the protein. Because several mutants of caveolin have previously been reported to show a similar subcellular distribution as the Cav1-GFP construct, we speculate that this phenotype may generally reflect the behavior of poorly folded forms of caveolin. Differences in the rate of transcription, translation, folding, stability, or resistance to degradation of EGFP and mCherry could potentially exacerbate folding defects of the protein (44, 53-58). Even though these defects are likely exaggerated under the conditions of our experiments, similar folding defects could potentially occur *in vivo* either when wild type caveolin is overexpressed or as the result of expression of mutant forms of the protein.

Consistent with previous reports that mutant forms of caveolin-1 exhibit defects in oligomerization and conformation when trapped intracellularly, we observed several significant changes in caveolin-1 epitope accessibility in cells expressing either Cav1-GFP or P132L Cav1-GFP, presumably as the result of the accumulation of abnormal oligomers and/or misfolded protein. Interestingly, some antibodies showed much more dramatic changes in accessibility than others, emphasizing the importance of using multiple antibodies to detect these shifts by immunofluorescence microscopy. The panel of antibodies described here should serve as a useful tool to identify additional conditions where caveolin-1 exists in aberrant conformations, thus extending current approaches to identify disease-related changes in the subcellular distribution, structure, and function of caveolin.

We also found that the perinuclear pool of Cav1-GFP is strongly recognized by a PTyr14 caveolin-1 antibody, raising the possibility that phosphorylation of the protein may contribute to this phenotype. Because the commercial PTry14 caveolin-1-antibody used here has been reported to cross-react with phosphopaxillin (51), we performed a number of control experiments to confirm that the PTyr14 antibody indeed recognizes phosphocaveolin-1 in the perinuclear compartment, not phosphopaxillin. The finding that perinuclear Cav1-GFP is phosphorylated on Tyr14 also prompted us to investigate the role of this phosphorylation event in this phenotype using a Cav1-GFP Y14F mutant. The localization of Y14F Cav1-GFP was indistinguishable from that of Cav1-GFP, indicating that phosphorylation is most likely a consequence, and not the cause of its defective trafficking. In addition, the Y14F mutant showed a similar dominant negative activity as Cav1-GFP, indicating that phosphorylation is not required for this behavior.

The signaling pathways that lead to Tyr14 phosphorylation of caveolin-1 when it is trapped intracellularly, and the physiological consequences of this aberrant caveolin-1 phosphorylation remain to be determined. We speculate that the changes in epitope accessibility of caveolin-1 under these conditions may provide enhanced access of Src to caveolin. Given these findings, in future studies, it will be of interest to determine whether enhanced caveolin-1 phosphorylation at Tyr14 can be used as a screening tool, especially given recent efforts to use caveolin-1 epithelial immunostaining patterns to stratify human breast cancer patients and predict the caveolin-1 P132L mutation (31).

Our findings have important implications for gain of function activity of mutant forms of caveolin-1 and diseases associated with caveolin-1 overexpression. The P132L mutant of caveolin-1 demonstrates both loss of function and gain of function activities, for reasons that are not yet entirely clear (32). Our current results provide several possible clues into the gain of function activity of this mutant. For example, changes in caveolin-1 conformation could not only interfere with caveolae assembly but also potentially impact the interactions of caveolin-1 with its binding partners throughout the cell. The accumulation of phosphorylated caveolin-1 in this compartment could also potentially recruit proteins that specifically bind tyrosine phosphorylated caveolin-1 (59, 60). Finally, our findings raise the possibility that overexpression of caveolin-1 may be sufficient to give rise to a dominant negative phenotype *in vivo* and that this may occur in a cell-type specific manner. Such a mechanism could potentially be important from a pathophysiological standpoint since caveolin-1 overexpression has been linked to several forms of cancer (61-64). More work will be required to test this hypothesis in future studies.

MATERIALS AND METHODS

Cell culture, antibodies, and plasmids

COS-7 cells, caveolin-1^{+/+} MEFs, caveolin-1^{-/-} MEFs, and HeLa cells were obtained from ATCC. Paxillin^{-/-} MEFs were kindly provided by Dr. Sheila Thomas (65). COS-7 and MEF cells were maintained in DMEM supplemented with 10% FCS in a humidified incubator at 37° C under 5% CO₂. HeLa cells were maintained in DMEM and supplemented with 12.5% Cosmic calf serum (Hyclone) at 37 °C and 5% CO₂. Cells were plated on glass coverslips 2 days prior to experiments. Where indicated, cells were transiently transfected with the indicated plasmids using FuGene6 (Roche Diagnostics, Indianapolis) or Effectene (Qiagen) the day prior to experiments. In most experiments, cells were transfected with a total of 1 µg of plasmid DNA.

Rabbit anti-caveolin-1 polyclonal antibody (designated here as pAb h1-97), mouse monoclonal antibody (mAb) clone 2297, mouse mAb clone 2234, mouse mAb clone C060, mouse mAb clone C20B, and mouse anti-phosphocaveolin Tyr14 were obtained from BD

Transduction Laboratories (San Jose, CA). Rabbit anti-c-terminal caveolin-1 mAb (mAb C-term) and rabbit anti-PTyr14 caveolin-1 mAb were obtained from Epitomics (Burlingame, CA). Anti-caveolin pAb N20 (sc-894) and rabbit anti-myc (sc-789) were from Santa Cruz Biotechnology Inc (Santa Cruz, CA). Mouse mAb anti-Golgin-97 was obtained from Invitrogen Molecular Probes (Carlsbad, CA). Rabbit anti-giantin was from Covance (Princeton, NJ). Rabbit anti-paxillin was from Abcam (Cambridge, MA). Rabbit phosphopaxillin Tyr118 pAb was from Cell Signaling Technology (Danvers, MA). Mouse anti-GFP was purchased from Invitrogen Molecular Probes (Carlsbad, CA). Fluorescently labeled secondary antibodies were purchased from Jackson ImmunoResearch Laboratories Inc. (West Grove, PA) and Invitrogen Molecular Probes (Carlsbad, CA).

Constructs

Cav1-GFP was kindly provided by Lucas Pelkmans and Ari Helenius (41). The Y14F mutant of Cav1-GFP was the gift of Mark McNiven (52). GalT-GFP was as described previously (66). Caveolin-1-myc was provided by Richard Anderson (45).

P132L Cav1-GFP was generated by site directed mutagenesis of Cav1-GFP according to the manufacturer's protocol (Stratagene) using the following primer set (Integrated DNA Technologies (IDT)): forward primer 5'CATCTGGGCAGTTGTACTATGCATTA3' and reverse primer 5'GGAAGCTCTTAATGCATAGTACAAC3'. Positive clones were identified by sequencing with a CMV Forward Primer (IDT). P132L-mCherry was generated from P132L-Cav1-GFP by a restriction enzyme digest with XhoI & BamHI and subsequent subcloning the Cav1 P132L into the mCherry vector.

To construct Cav1-mCherry, a caveolin-1 fragment was generated by a restriction enzyme digest with Hind III and Apa I (Promega, Madison, WI) from Cav1-GFP (Pelkmans et al., 2001). The resulting fragment was subcloned into a mCherry N1 vector and a positive clone of Cav1-mCherry was identified by sequencing.

Cav1-mGFP was generated by site directed mutagenesis of EGFP to include the A206K mutation (42) in the Cav1-GFP construct according to the manufacturer's protocol (Stratagene) using the following primer set (Sigma): forward primer 5'AGCACCCAGTCCAACTGAGCAAAGACCCC3' and reverse primer 5'GGGGTCTTTGCTCAGTTTGGACTGGGTGCT3'. Positive clones were identified by sequencing with a GFP Forward sequencing primer (Sigma).

Cav1-mEmerald was generated using human caveolin-1 (Origene, Rockville, MD, U.S.A.; NM_001753). It was amplified with a 5' primer encoding a NheI site as well as a kozack sequence and a 3' primer encoding a BamHI site. The digested fragments were then ligated into a similarly cut N1 (Clontech, Mountain View, CA) cloning vector containing the EGFP derivative mEmerald. Positive clones were identified by sequencing.

Immunofluorescence staining

COS-7 and MEF cells were fixed in 3.7% PFA for 15 min at RT. After rinsing several times in PBS they were blocked for 15 min in PBS containing 10% FCS and 0.1% saponin for 30 min. Antibody dilutions were performed in blocking solution containing 0.1% saponin. Cells were incubated with primary antibodies for 30 min at RT. Final dilutions were prepared in blocking solution as follows: pAb h1-97 was used at 1:300, pAb N20 at 1:200, mAb 2297 at 1:200, mAb C-term at 1:100, mAb 2234 at 1:50, mAb C060 at 1:50, mAb C20B at 1:50, mAb GFP at 1:50, rabbit anti-myc at 1:400, mouse anti-phosphoTyr14 caveolin-1 at 1:50, rabbit phosphoTyr14 caveolin-1 at 1:100, rabbit phospho-paxillin (Tyr118) at 1:50, and golgin-97 at 1:200. Cells were then washed 3 × 5 min in PBS, and incubated with 1 µg/ml secondary antibodies for 30 min at RT, and washed again. Coverslips were then either

mounted on slides using Fluormount G (Southern Biotech, Birmingham, AL) and sealed with nailpolish prior to imaging, or mounted in Fluoromount G supplemented with 25 mg/ml DABCO (1,4 diazabicyclo[2.2.2]octane) (Sigma-Aldrich) and allowed to solidify overnight prior to imaging.

HeLa cells were fixed in 3.7% PFA in for 15 minutes at 37° C. After fixation, the coverslips were washed three times with PBS for 5 minutes and then permeabilized with 0.2% Triton X-100 in PBS for 1 hour on an orbital shaker. After permeabilization the cells were washed three times with 0.05% Triton X-100 in PBS for 5 minute and then blocked in 10% Normal Goat Serum/0.05% Triton X-100 in PBS for 1 hour. Cells were incubated with primary antibody for 1.5 h at 37° C. Following incubation, the coverslips were washed with blocking solution three times for 10 minutes and then incubated with secondary antibody for 37°C for 1 hour. The cells were then washed with 1% Normal Goat Serum/0.05% Triton X-100 in PBS three times for 10 minutes, then washed three times with PBS for 5 min. Coverslips were mounted with gelvatol and imaged the following day.

Fluorescence microscopy

Confocal images of COS-7 and MEF cells were obtained using a Zeiss LSM 510 confocal microscope (Carl Zeiss, Thornwood, NY) with a 40× 1.3 NA Plan Neofluar or 100× 1.4 NA Plan Apochromat objective and standard filter sets provided by the manufacturer. Images were collected with the confocal pinhole set at 1-1.5 Airy units. For presentation purposes, images were exported as tiff files and Adobe Photoshop was used to convert the images to grayscale and adjust image contrast.

Fluorescence images of HeLa cells were acquired with a Nikon 80i upright microscope coupled to a Hamamatsu Orca ER camera using a 60× 1.4 NA Nikon Plan Apochromat Objective and Chroma FITC and Texas Red filter sets. Each fluorophore was excited and imaged independently. Colocalization analysis was performed using Nikon imaging software (NIS-Elements AR version 3.00).

Analysis of caveolin-1 expression levels

COS-7 and HeLa cells were plated in 6-well plates. A single coverslip was included in each well. The following day the cells were transfected with either empty EGFP vector, empty mCherry vector, Cav1-GFP, or Cav1-mCherry. 24 hours later, the coverslips were removed and fixed for 15 minutes in 4% PFA. Cell nuclei were labeled using 1:1000 DRAQ5 (Cell Signaling Technology Inc.) for 5 minutes at room temperature. The plasma membrane was labeled with 200 nM of either Alexa555-CTxB or Alexa488-CTxB (Life Technologies Corp.) for 5 minutes at room temperature, washed and then mounted on a live cell chamber filled with PBS.

Coverslips were imaged on a Zeiss LSM 510 using a Plan-Neofluar 10× 0.3 NA objective. 2040 × 2040 pixel images were acquired at 1× digital zoom in three different channels: green, red, and far red using a 488 laser line, a 543 laser line, and a 633 laser line and filter sets provided by the manufacturer. To calculate the transfection efficiency, a custom-written ImageJ macro (available upon request) was used to identify nuclei and score the cells as either positive or negative for Cav1 fluorescence.

The remaining cells were harvested by lysing in sample buffer and boiling for 10 min. Equal amounts of protein (30 µg) were loaded for each lysate on a 4-12% SDS-PAGE gel prior to western blotting. Blots were probed with the caveolin-1 antibody pAb h1-97 at 1:10,000 dilution and IRDye 800CW conjugated goat polyclonal anti-rabbit IgG (H+L) (LI-COR Biosciences, Lincoln, Nebraska) at 1:10,000 dilution. The fluorescent signal was detected with LI-COR Odyssey infrared imaging system (LI-COR Biosciences, Lincoln, Nebraska).

Quantification of the western blot images was performed using Image J and normalized for transfection efficiency to calculate the fold overexpression relative to endogenous caveolin-1.

Analysis of dominant negative phenotype

Cells were analyzed using Image J software. In cells expressing Cav1-mCherry, the ratio of perinuclear to peripheral fluorescence was calculated from regions of interest drawn around the perinuclear and peripheral regions of the cell as illustrated in the inset in Figure 4. In cells expressing Cav1-myc, the area of the perinuclear region was determined by drawing bisecting lines through the region and multiplying their lengths to obtain the area in pixels squared.

Cycloheximide treatment

For cycloheximide treatment, cells were incubated at 37° C with 200 µg/ml cycloheximide (Sigma-Aldrich) for 5 (Cav1-GFP fusions) or 16 h (endogenous caveolin-1) prior to fixation and processing for immunofluorescence or imaging of GFP fluorescence.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Drs. Lucas Pelkmans, Mark McNiven, James Goldenring, and Richard Anderson for providing constructs, Dr. Sheila Thomas for the paxillin^{-/-} MEFs, Dr. William Bush for assistance at early stages of this study, Paul Barrett for help with data analysis, Courtney Copeland for assistance with experiments, and Drs. Lynne Lapierre and Debbie Brown for helpful discussions. This work was supported by R01 GM073846 and R01 HL111259 from the NIH. The content is solely the responsibility of the authors and does not necessarily represent the official views of the NIGMS or the NIH.

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Figure 1. Cav1-GFP accumulates in a perinuclear compartment in COS-7 cells, similar to the subcellular distribution of a disease associated caveolin-1 mutant, P132L

Representative phenotypes of COS-7 cells expressing (A) P132L-Cav1-GFP, (B, C) Cav1-GFP, (D) P132L Cav1-mCherry, (E, F) Cav1-mCherry, (G) Cav1-mGFP, (H) Cav1-mEmerald, or (I) Cav1-myc. In panel C, arrows point to examples of a subset of cells in which Cav1-GFP is present in discrete puncta instead of clustered in a perinuclear compartment. In panel I, arrows point to cells with predominantly diffuse ER-like Cav1-myc staining patterns, and arrowheads point to cells with both diffuse plasma membrane staining and perinuclear Cav1-myc. In panels A-I, cells were transiently transfected with 1 µg of the indicated construct and either imaged live (A-H) or fixed and immunostained prior to imaging (I). (J, K) Representative phenotypes of COS-7 cells transfected with 0.1 µg of the Cav1-GFP construct and 0.9 µg of mCherry (empty vector). Cells were imaged live. (L) Effect of expression level of Cav1-GFP on the subcellular distribution of the protein. Cells were transfected with a mixture of Cav1-GFP and empty mCherry plasmid DNA to yield a total of 1 µg. The amount of Cav1-GFP plasmid DNA used is indicated on the x-axis. The percentage of cells with either a perinuclear accumulation of Cav1-GFP similar to that shown in panels B and J (black bars) or punctate Cav1-GFP staining similar to that shown in panel K (gray bars) were quantified across 3 independent experiments (N = 60 to 82 cells total per condition). Bar = 10 µm.

Figure 2. The extent of perinuclear accumulation of overexpressed Cav1 depends on the cellular context

HeLa cells were transiently transfected with each of the indicated constructs, fixed, and immunostained for giantin. Representative phenotypes are shown for cells expressing (A) P132L Cav1-GFP, (B) Cav1-GFP, (C) Cav1-mCherry, and (D) Cav1-mEmerald. Bar = 10 μ m.

Figure 3. The extent of perinuclear accumulation of overexpressed Cav1 does not depend on the levels of endogenous caveolin-1 expression

(A) Caveolin-1^{+/+} and (B) caveolin-1^{-/-} MEFs cells were transiently transfected with the indicated constructs and imaged live. Representative phenotypes are shown. Bar = 10 μm.

Figure 4. Cav1-GFP functions as a dominant negative in COS-7 cells

COS-7 cells were cotransfected with the indicated constructs. The following day, cells were fixed and imaged directly, or immunostained using an anti-myc antibody prior to imaging. **(A)** Representative images of cells coexpressing Cav1-mCherry and either EGFP, Cav1-GFP, or P132L Cav1-GFP. In the merged images, mCherry fluorescence is shown in red and GFP fluorescence in green. Bar, 10 μ m. **(B)** Representative images of cells coexpressing Cav1-myc and EGFP, Cav1-GFP, or P132L Cav1-GFP. In the merged images, myc immunostaining is shown in red and GFP fluorescence in green. Bar, 10 μ m. **(C)** Quantification of the ratio of Cav1-mCherry fluorescence in the perinuclear region versus cell periphery in cells coexpressing Cav1-mCherry and EGFP, Cav1-GFP, or P132L Cav1-GFP. An example of the regions used for image analysis is shown in the inset. **(D)** Quantification of the area of the perinuclear caveolin-1-myc compartment in cells coexpressing Cav1-myc and EGFP, Cav1-GFP, or P132L Cav1-GFP. An example of the regions used for quantitative analysis is shown in the inset. Data in (C) and (D) are from a single experiment including 15-22 cells per condition and are representative of three independent experiments. Error bars, SE. *, $p < 0.05$, Student T test.

Figure 5. Cav1-GFP and Cav1-GFP P132L are chased out of the perinuclear region following inhibition of new protein synthesis

(A) COS-7 cells were either left untreated or incubated with 200 $\mu\text{g/ml}$ CHX for 16 h. They were then fixed and stained for endogenous caveolin-1 using a rabbit polyclonal antibody (h1-97) or golgin-97. (B) Quantification of the loss of endogenous perinuclear caveolin-1 and golgin-97 following CHX treatment. Data are from a single experiment including 25-70 cells per condition and are representative of three independent experiments. (C) COS-7 cells were transfected with Cav1-GFP or P132L Cav1-GFP. The following day, they were either left untreated or treated with 200 $\mu\text{g/ml}$ CHX for 5 h prior to fixation and imaging. (D) Quantification of the loss of perinuclear Cav1-GFP and P132L Cav1-GFP following CHX treatment. Data are taken from a single experiment including 9-15 cells per condition and are representative of three independent experiments. Bar, 10 μm . *, $p < 0.05$, Student T test.

Figure 6. The epitope accessibility of caveolin-1 is substantially altered in cells expressing Cav1-GFP or P132L Cav1-GFP

(A) COS-7 cells were fixed in PFA, permeabilized with saponin, and immunostained with the indicated caveolin-1 antibodies. (B) Epitope accessibility of caveolin-1 in COS-7 cells expressing Cav1-GFP (C) Epitope accessibility of caveolin-1 in COS-7 cells expressing Cav1-GFP P132L. In the merged images, antibody staining is shown in red and caveolin-1 fluorescence in green. Bar, 10 μ m.

Figure 7. Perinuclear Cav1-GFP is phosphorylated on Tyr14

(A-D) mAb PTyr14 caveolin-1 immunostaining in (A) COS-7, (B) caveolin-1^{+/+} MEFs, (C) caveolin-1^{-/-} MEFs, and (D) paxillin^{-/-} MEFs. (E) COS-7 cells expressing Cav1-GFP were immunostained with a mouse monoclonal anti-PTyr14 cav1 antibody. (F) mAb PTyr14 caveolin-1 staining in COS-7 cells expressing P132L Cav1-GFP. (G) Paxillin immunostaining in COS-7 cells expressing Cav1-GFP. (H) COS-7 cells expressing Cav1-GFP were immunostained with an antibody. (I) Anti-PY118 paxillin immunostaining in COS-7 cells expressing Y14F Cav1-GFP. In the merged images, antibody staining is shown in red and GFP fluorescence in green. Bar, 10 μ m.

Figure 8. Phosphorylation of caveolin-1 at Tyr14 is not required for intracellular accumulation of the protein or for its dominant negative activity

(A) Representative images of a cell coexpressing Y14F Cav1-GFP (green) and Cav1-mCherry (red). (B) Representative images of a cell coexpressing Y14F Cav1-GFP (green) and Cav1-myc (red). (C) Quantification of the ratio of Cav1-mCherry fluorescence in the perinuclear region versus cell periphery in cells coexpressing Cav1-mCherry and EGFP, Cav1-GFP, or Y14F Cav1-GFP. Mean $\pm$ SE are shown for N = 12-18 cells from a representative experiment of 3 independent experiments. *, $p < 0.05$, Student T test. (D) Quantification of the size of the perinuclear caveolin-1-myc compartment in cells coexpressing Cav1-myc and EGFP, Cav1-GFP, or Y14F Cav1-GFP. Mean $\pm$ SE are shown for N = 14-16 cells from a representative experiment of 3 independent experiments. *, $p < 0.05$, Student T test. (E) COS-7 cells transfected with Y14F Cav1-GFP were immunostained with the indicated caveolin-1 antibodies. In the merged images, antibody staining is shown in red and caveolin-1 fluorescence in green. Bar, 10 μ m.

Table 1

Caveolin-1 constructs used in this study

Construct	Linker sequence	Species	Reference
Cav1-GFP	Cav1- <i>P-R-A-R-D-P-P-V-A-T</i> -EGFP	<i>C. familiaris</i>	(41)
P132L Cav1-GFP ^I	P132L Cav1- <i>P-R-A-R-D-P-P-V-A-T</i> -EGFP	<i>C. familiaris</i>	This study
Cav1-mGFP ^I	Cav1- <i>P-R-A-R-D-P-P-V-A-T</i> -mEGFP	<i>C. familiaris</i>	This study
Cav1-mCherry ^I	Cav1- <i>P-R-A-R-D-P-P-V-A-T</i> -mCherry	<i>C. familiaris</i>	This study
P132L Cav1-mCherry ^I	P132L Cav1- <i>P-R-A-R-D-P-P-V-A-T</i> -mCherry	<i>C. familiaris</i>	This study
Cav1-mEmerald	Cav1- <i>S-S-G-G-D-P-P-V-A-T</i> -mEmerald	<i>H. sapiens</i>	This study
Cav1-myc	Cav1- <i>L-G-P</i> (myc)- <i>N-I-A-V-D-H-H-H-H-H</i>	<i>H. sapiens</i>	(45)
Cav1-GFP Y14F	Y14F Cav1- <i>V-T-F-Q-G-I-L-Q-S-T-V-P-R-A-R-D-P-P-V-A-T</i> -EGFP	<i>R. norvegicus</i>	(52)

^IGenerated from the Cav1-GFP construct described in (41)

Caveolin-1 antibodies used in this study^{1,2}

Designation	Catalog #	Epitope	Isoform specificity
pAb h1-97	610059	Epitope mapped to residues 1-21 and 61-101 ³	$\alpha + \beta$
mAb 2234	610493	Epitope mapped to residues 1-24 ⁴	α
mAb 2297	610406	Epitope mapped to residues 61-71 ⁴	$\alpha + \beta$
pAb N20	sc-894	N-terminus (human)	α
C-term	1249-1	near C-terminus (human)	$\alpha + \beta$
mAb PY14	611338	phosphotyrosine 14 ⁵	α
pAb PY14	2267-1	phosphotyrosine 14	α

¹ All antibodies were obtained from BD Biosciences with the exception of pAb N20 (Santa Cruz Biosciences), C-term (Epitomics), and pAb PY14 (Epitomics).

² In the current study, mAb C060 and mAb C20B (BD Biosciences) were found to crossreact with an unknown protein or proteins in caveolin-1^{-/-} MEFs by immunofluorescence and thus were not used for further study.

³ Epitope mapped in (67).

⁴ Epitope mapped in (19)

⁵ This antibody has been reported to crossreact with phosphopaxillin (51)