Caveolin Regulates Endocytosis of the Muscle Repair Protein, Dysferlin*5

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Dysferlin and Caveolin-3 are plasma membrane proteins associated with muscular dystrophy. Patients with mutations in the CAV3 gene show dysferlin mislocalization in muscle cells. By utilizing caveolin-null cells, expression of caveolin mutants, and different mutants of dysferlin, we have dissected the site of action of caveolin with respect to dysferlin trafficking pathways. We now show that Caveolin-1 or -3 can facilitate exit of a dysferlin mutant that accumulates in the Golgi complex of Cav1−/− cells. In contrast, wild type dysferlin reaches the plasma membrane but is rapidly endocytosed in Cav1−/− cells. We demonstrate that the primary effect of caveolin is to cause surface retention of dysferlin. Caveolin-1 or Caveolin-3, but not specific caveolin mutants, inhibit endocytosis of dysferlin through a clathrin-independent pathway colocalizing with internalized glycosylphosphatidylinositol-anchored proteins. Our results provide new insights into the role of this endocytic pathway in surface remodeling of specific surface components. In addition, they highlight a novel mechanism of action of caveolins relevant to the pathogenic mechanisms underlying caveolin-associated disease.

Dysferlin and Caveolin-3 (muscle-specific caveolin, Cav3) are sarcosomal proteins whose role in muscle has gained clinical attention because mutations in their genes are associated with a number of muscle pathologies. Patients with mutations in the dysferlin (DYSF) gene develop disorders such as limb girdle muscular dystrophy type 2B, Miyoshi myopathy, and distal myopathy (1–5). Whereas disruption in the Caveolin-3 (CAV3) gene show dysferlin mislocalization in muscle cells. We demonstrate that the primary effect of caveolin is to cause surface retention of dysferlin. Caveolin-1 or Caveolin-3, but not specific caveolin mutants, inhibit endocytosis of dysferlin through a clathrin-independent pathway colocalizing with internalized glycosylphosphatidylinositol-anchored proteins. Our results provide new insights into the role of this endocytic pathway in surface remodeling of specific surface components. In addition, they highlight a novel mechanism of action of caveolins relevant to the pathogenic mechanisms underlying caveolin-associated disease.

The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1 and S2.

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The abbreviations used are: PM, plasma membrane; Cav, Caveolin; Cav1−/−, Cav3−/−, immortalized cell lines; CSD, caveolin scaffolding domain; CTB, cholera toxin binding subunit; GPI-AP, glycosylphosphatidylinositol-anchored proteins; GEECs, GPI-AP-enriched early endosomal compartments; Tfn, transferrin; WT, wild type; AA, ascorbic acid; HA, hemagglutinin; GFP, green fluorescent protein; HRP, horseradish peroxidase; MEF, mouse embryonic fibroblasts; DAB, dimethylaminobenzene; IC, intracellular; TM, transmembrane.

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domain (CSD), has been shown to bind in vitro to a consensus sequence \((\phi \chi \phi \chi \chi \phi \chi \chi \phi, \phi \text{ aromatic residues, } X \text{ any amino acid})\) (46) present in various proteins. Dysferlin has several putative CSD binding motifs (17).

We have recently described the subcellular distribution of dysferlin with respect to Cav3 and showed that dysferlin association with the PM is impaired in the absence of caveolin, or in the presence of dystrophy-associated mutant forms of Cav3 (18). Although Cav3 and dysferlin copurify (16, 17), the precise interacting domains and roles in their trafficking dynamics are poorly understood. We show here that in the absence of caveolin, dysferlin reaches the PM but is rapidly endocytosed through a caveolin-, clathrin-, and dynamin-independent pathway. Wild type caveolin, but not mutant forms of caveolin associated with muscle disease, specifically inhibit dysferlin endocytosis causing its retention at the cell surface.

**EXPERIMENTAL PROCEDURES**

**DNA Constructs, Reagents, and Antibodies**—Cell culture reagents were obtained from Invitrogen. The antibodies used were: mouse anti-LBPA, mouse anti-LAMP-1 (Southern Bio tech), mouse anti-GM130 (BD Biosciences), rabbit antibody made against the conserved region of Cav3 (47), rabbit anti-HA (Dr. T. Nilsson, Gothenburg University, Gothenburg, Sweden), rabbit anti-GFP (48), mouse anti-myc 9B11 (Cell Signaling Technology), and anti-protein-disulfide isomerase. Secondary antibodies conjugated to Alexa Fluor 555, or 5 mg/ml Tfn Alexa Fluor 647 was bound to cells on ice for 30 min in CO\(_2\)-independent medium. Cells were washed with ice-cold CO\(_2\)-independent medium to remove unbound reagent prior to uptake in growth media (10% fetal bovine serum, 2 \(\text{mM L-glutamine/Dulbecco’s modified Eagle’s medium}\) at 37 °C, for the times indicated. Cells were plated on cold CO\(_2\)-independent medium and washed 2 times for 30 s in 0.5 mM glycine (pH 2.2). The cells were fixed in 2% paraformaldehyde and processed for immunofluorescence. For inhibition of dynamin-dependent uptake, cells were preincubated in either 80 mM dynasore/growth media or 0.4% Me\(_2\)SO/growth media for 30 min at 37 °C, followed by Tfn and anti-myc uptake in the presence or absence of dynasore.

**Ultrastructural Analysis of Dysferlin Endocytosis in WT Cav1 and Cav1\(^{-/-}\) MEFs**—WT Cav1 or Cav1\(^{-/-}\) MEFs were transfected with GFP dysferlin. After overnight incubation, to allow expression of the constructs, the cells were incubated with mouse anti-myc antibodies at 4 °C for 20 min, washed, and further incubated with anti-mouse HRP at 4 °C for 20 min. The cells were warmed to 37 °C for 2 min to allow uptake and then incubated in DAB, with or without ascorbic acid (AA), fixed, and processed for resin embedding, exactly as described previously (54). Due to the low transfection efficiency, GFP-expressing cells were identified by light microscopy before processing. They were marked to allow subsequent location for sectioning. Quantitation of PM coverage of the HRP reaction product was by intersection counting. The number of intersections of a square lattice grid with unlabeled and DAB-covered areas of the PM in random areas of transfected WT Cav1 or Cav1\(^{-/-}\) MEFs was measured on digital images to gain an estimate of PM coverage by the HRP reaction.

**RESULTS**

**Subcellular Distribution of Dysferlin Truncation Mutants in WT Cav1 and Cav1\(^{-/-}\) MEF Cells**—To better understand the functional link between dysferlin and Cav3 we examined the subcellular distribution of truncation mutants of dysferlin and analyzed their trafficking dependence on caveolin. We used WT Cav1 and Cav1\(^{-/-}\) MEFs as a model system. Cav1\(^{-/-}\) cells have no detectable caveolae as they lack Cav1 (as well as the
muscle-specific isoform Cav3). This represents a powerful model system to analyze dysferlin trafficking with respect to caveolin as caveolin re-expression rescues the dysferlin trafficking defects (18).

Truncated versions of dysferlin with an N-terminal GFP tag were generated (see summary in Fig. 1). Expression of these mutants in baby hamster kidney cells showed single bands of the predicted molecular weight for each of the truncation mutants (Fig. 1B). We compared the subcellular distribution of the mutant proteins to the WT protein by heterologous expression of truncation mutant forms of dysferlin in WT Cav1 or Cav1/H11002 MEFs (refer to summary table in Fig. 1). Full-length dysferlin efficiently reaches the PM in WT Cav1 MEFs (Fig. 1C), consistent with previous results (18). In contrast, dysferlin localized to intracellular structures but not to the Golgi complex in Cav1/H11002 cells (Fig. 1C). GFP/H9004-C2, which lacks the three first C2 domains, was found to mainly localize to the endoplasmic reticulum as judged by a large overlap with the endoplasmic reticulum marker, protein-disulfide isomerase (Fig. 1, C–E). Truncated versions of dysferlin lacking the TM domain, GFPΔ-3 and GFPΔ-TM, were however, mainly cytosolic (Fig. 1, C–E) and were indistinguishable in WT Cav1 and Cav1/H11002 cells.

To examine whether this reflected a direct role of caveolin in facilitating the transport of GFPΔ-1 to the PM, we co-expressed GFPΔ-1 and HA-tagged Cav1 (Cav1-HA) or Cav3 (Cav3-HA) in Cav1/H11002 MEFs. GFPΔ-1 efficiently exited the Golgi apparatus and reached the PM (Fig. 2). Quantitation showed that in ~66% of Cav1/H11002 MEFs expressing...
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Cav1-HA or Cav3-HA, GFPΔ-1 was localized to the PM and to intracellular puncta (Fig. 2B). GFPΔ-C2, a mutant lacking all six C2 domains was predominantly targeted to the PM in both WT Cav1 (not shown) and Cav1<sup>−/−</sup> MEFs (Figs. 1, C–E, and 3, A and B). Quantitation of Cav1<sup>−/−</sup> MEFs expressing GFPΔ-C2 showed that in ≈97% of the cells, GFPΔ-C2 localized to the PM.

Taken together these results show that GFPΔ-1, which lacks the first four C2 domains, predominantly accumulates in the Golgi complex in the absence of caveolin. This suggests that caveolin is required for GFPΔ-1 transport from the Golgi complex to the PM. In contrast, a mutant lacking all six C2 domains is not retained in the Golgi complex in the presence or absence of caveolin. The loss of all six C2 domains renders mutated dysferlin independent of caveolin for surface delivery.

The fact that GFPΔ-C2 efficiently reached the PM in Cav1<sup>−/−</sup> MEFs (Fig. 3) showed that this truncated protein was not dependent on caveolin for surface targeting. We investigated whether a Golgi-localized dystrophy mutant of Cav3 (Cav3P104L-HA), which causes retention of full-length dysferlin in the Golgi complex (18) would affect the traffic of GFPΔ-C2 to the PM. Cells co-expressing epitope-tagged Cav3P104L (Cav3P104L-HA) and GFPΔ-C2 showed a dramatic accumulation in the Golgi complex (66% of the cells) compared with cells expressing mutated dysferlin alone (4% of the cells) (Fig. 3). Thus GFPΔ-C2, which does not require caveolin for Golgi exit and PM targeting, is blocked in trafficking from the Golgi by the P104L caveolin mutant.

Characterization of Dysferlin Trafficking in Cells Lacking Caveolin—

In the absence of caveolin dysferlin accumulates in an intracellular compartment of unknown nature. The identification of these structures should provide insights into the role of caveolins in dysferlin trafficking. We first examined whether dysferlin was targeted for degradation in Cav1<sup>−/−</sup> cells. However, dysferlin failed to co-localize significantly with markers of the late endocytic pathway such as LBPA and LAMP1 (supplemental Fig. S1A). Despite the low surface labeling in the Cav1<sup>−/−</sup> MEFs, we speculated that dysferlin is able to reach the PM but then is efficiently endocytosed in the absence of caveolin. If this was the case, antibodies to the luminal myc epitope should be readily internalized by Cav1<sup>−/−</sup> cells expressing GFPDysf but not by WT Cav1 cells. WT Cav1 and Cav1<sup>−/−</sup> MEFs were transfected with a dysferlin cDNA containing an N-terminal GFP tag and a C-terminal (luminal/extracellular) myc tag (GFPDysf). After 4 h post-transfection, antibodies against the dysferlin ectoplasmic myc tag were added to the culture medium and antibodies were allowed to internalize overnight. The cells were then fixed, permeabilized, and labeled with secondary antibodies. A striking accumulation
of myc antibodies was observed in the Cav1<sup>−/−</sup> MEFs (Fig. 4A), very little was internalized in WT Cav1 and no uptake was observed in neighboring non-transfected cells (see Fig. 4A) indicating that the antibodies were taken up specifically after binding to the exposed lumenal myc epitope and not by fluid phase uptake. Consistent with this, the internalized antibodies colocalized with GDPDysf. Identical results were obtained when experiments were performed using Fab fragments against the myc epitope (supplemental Fig. S1B). Cav1<sup>−/−</sup> MEFs expressing GFP-dystf showed higher uptake of Fab fragments compared with WT Cav1 cells (supplemental Fig. S1B). There was no colocalization of Fab fragments and transferrin in WT Cav1<sup>−/−</sup> or Cav1<sup>−/−</sup> cells at any of the time points examined (data not shown).

To investigate this in more detail, myc antibodies were bound to the surface of GDPDysf expressing Cav1<sup>−/−</sup> or WT Cav1 cells at 4 °C and warmed for 30 min at 37 °C to allow internalization of the antibodies. Surface antibodies were removed by an acid wash. Interestingly, in WT Cav1 or Cav1<sup>−/−</sup> MEFs, significant GDPDysf internalization was observed after 30 min. Despite the low level of PM dysferlin, Cav1<sup>−/−</sup> MEFs contained many more vesicles positive for both GFP and myc (Fig. 4B) suggesting a much higher endocytic rate. This was confirmed by quantitation of the intracellular/PM (IC/PM) ratio after uptake at 10 min (WT Cav1, IC/PM ratio = 0.05 ± 0.001; Cav1<sup>−/−</sup>, IC/PM ratio = 0.52 ± 0.04) and 40 min (WT Cav1, IC/PM ratio = 0.10 ± 0.009; Cav1<sup>−/−</sup>, IC/PM ratio = 1.07 ± 0.12) (Fig. 4C). Furthermore, and consistent with our previous work (18), we predicted that re-expression of Cav1-1A in Cav1<sup>−/−</sup> MEFs would inhibit dysferlin internalization (Fig. 4D). Cav1<sup>−/−</sup> MEFs co-expressing GFPDysf and Cav1-1A showed a distribution of dysferlin similar to that seen in WT Cav1 MEFs (refer to supplemental Fig. S2A; the ability of dysferlin to reside at the PM membrane has been rescued by expression of Cav-1 (Fig. 4D and supplemental Fig. S2A). This was further confirmed by quantifying the amount of internalized myc antibodies in WT Cav1 and Cav1<sup>−/−</sup> MEFs expressing GDPDysf (WT Cav1, 9.06 ± 0.67; Cav1<sup>−/−</sup>, 24.04 ± 1.89) or Cav1<sup>−/−</sup> cells co-expressing GFP-dysf and Cav1-1A (9.33 ± 0.56) (refer to “Experimental Procedures”) (Fig. 4D). Thus these results demonstrate that the major effect of caveolin is to retain dysferlin at the PM and inhibit its internalization. These results demonstrate that dysferlin is not absolutely dependent on caveolin to reach the PM but is efficiently retained at the PM in the presence of caveolin. Taken together, these results show a hitherto unexpected dynamic cycling of dysferlin in Cav1<sup>−/−</sup> cells.

Dysferlin Is Internalized Through a Clathrin-independent Endocytic Pathway—We next examined the pathway by which dysferlin is internalized in Cav1<sup>−/−</sup> MEFs using transferrin to label the clathrin pathway and cholera toxin binding subunit (CTB) or GPI-anchored proteins (AP) as markers of other pathways. Myc antibodies taken up by expressed GFP-dysferlin for various times did not colocalize significantly with transferrin (Fig. 5A). However, significant colocalization of internalized myc antibodies and CTB was evident after 2, 10, and 40 min of internalization (Fig. 5A). No significant difference in the internalization rate of transferrin or CTB was seen between WT Cav1 or Cav1<sup>−/−</sup> MEFs (supplemental Fig. S2B).

GPI-AP are internalized via a clathrin- and dynamin-independent endocytic pathway (54–56). To test if dysferlin was trafficking from the PM via this pathway we co-internalized antibodies against the extracellular tags (mouse anti-myc for dysferlin and rabbit anti-GFP for GPI-GFP). Cav1<sup>−/−</sup> MEFs co-expressing GFPDysf and GPI-GFP were labeled on ice with anti-myc and anti-GFP antibodies, warmed to 37 °C for 2, 10, and 40 min, and then acid-washed. Internalized antibodies...
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Dysferlin cycles between an intracellular compartment and the plasma membrane. WT Cav1 and Cav1−/− MEFs were transfected with GFP·Dysf and allowed to internalized anti-myc antibodies overnight or for 30 min at 37 °C. A, in both WT and Cav1−/− cells dysferlin is cycling between the PM and an intracellular endocytic compartment. Inset shows extensive colocalization between GFP·Dysf and internalized myc antibodies in Cav1−/− cells (*, untransfected cell; **, cell expressing low levels of dysferlin) but in WT Cav1 cells there is very little internalization. B, in WT Cav1 MEFs dysferlin is mainly localized at the PM although some internalized myc can be seen after 30 min (see inset). In contrast after 30 min of myc antibody internalization, dysferlin shows a highly dynamic endocytic traffic in Cav1−/− cells; inset, extensive overlay between GFP·Dysf and internalized myc. C, time course of myc antibodies uptake in WT or Cav1−/− MEFs expressing GFP·Dysf. The mean fluorescence intensity of dysferlin associated with the PM (myc surface labeling) and internalized myc (2, 10, and 40 min chase at 37 °C) was measured and expressed as IC/PM ratio. D, dysferlin internalization is rescued to WT levels by expression of epitope-tagged Cav1 in Cav1−/− MEFs. WT Cav1 and Cav1−/− MEFs were transfected with GFP·Dysf or co-transfected with GFP·Dysf and Cav1-HA and anti-myc antibodies were internalized for 20 min at 37 °C. The mean fluorescence intensity of internalized dysferlin (myc labeling) was quantified. Error bars are S.E. of three experiments (n = 30). Bars, 10 μm.

were detected with anti-mouse Alexa Fluor 546 and anti-rabbit Alexa Fluor 647 antibodies after permeabilization. At all time points endocytic vesicles containing myc and GFP antibodies were readily detectable (Fig. 5A), demonstrating that internalized dysferlin was targeted to a GPI-AP enriched compartment. We further investigated if dysferlin vesicular traffic followed a dynamin-dependent route using the dynamin inhibitor, Dynasore (57, 58). Whereas transferrin uptake was blocked, dysferlin endocytosis was unaffected by incubation with Dynasore (Fig. 5B). We conclude that the major endocytic pathway involved in dysferlin endocytosis in Cav1−/− cells is dynamin-independent.

Ultrastructural Analysis of Dysferlin Trafficking—To gain further insights into dysferlin endocytosis, Cav1−/− or WT Cav1 MEFs were transfected with GFP·dysf, then incubated with anti-myc antibodies followed by an anti-mouse HRP-labeled antibody at 4 °C. The cells were then warmed for 2 min at 37 °C, and the DAB reaction visualized in the presence or absence of AA to identify internal structures, as in previous studies (54). The cells were then fixed and processed for correlative light and electron microscopy, identifying GFP·dysf-expressing cells by light microscopy and then sectioning the plastic embedded cells for EM. Consistent with the light microscopy, WT cells showed a uniform, almost continuous, layer of HRP reaction product over the entire cell surface (WT Cav1 − AA; Fig. 6, A and B) and little internal staining. No preferential staining of caveolae was observed, consistent with our previous immunoelectronmicrograph studies (18). In contrast, Cav1−/− MEFs showed very patchy sparse labeling over the cell surface (KO − AA; Fig. 6, C and D) but with some tubular profiles apparently enriched in reaction product (Fig. 6, E and G). Quantitation of the surface coverage of the HRP reaction product in WT Cav1 versus Cav1−/− cells showed a far higher surface coverage in the WT Cav1 cells (see Fig. 6, A and C) consistent with the low level of surface labeling in Cav1−/− cells as observed by light microscopy. Endocytic structures were clearly observed in the Cav1−/− cells treated with AA (KO + AA, Fig. 6, H and I). The ring-shaped morphology and size of the labeled elements are consistent with
structures labeled by CTB HRP in WT and Cav1−/− MEFs in the previous studies (54). These studies show that dysferlin is retained over the entire cell surface in the presence of caveolin but is rapidly internalized in its absence.

**Dysferlin Trafficking Is Not Rescued by Caveolin Scaffolding Domain Mutants in Cav1−/− MEF Cells**—To gain further insights into the functional interaction between dysferlin and caveolin and the relevance of these observations to muscle disease, we examined the effect of caveolin mutants on dysferlin endocytosis as compared with wild type caveolin. We made use of HA-tagged CSD point mutants, Cav3G55S (Cav3G55S-HA) and Cav3C71W (Cav3C71W-HA) (50, 59), which have been linked to muscular dystrophy (11, 13) and the CSD deletion mutant, Cav1Δ81–100 (Cav1Δ-81–100-HA) (60). Quantitation of surface versus intracellular dysferlin was performed using antibodies to the luminal myc tag as in previous studies (18). Cav3G55S-HA or Cav3C71W-HA expressed in Cav1−/− MEFs localized predominantly at the surface in a similar fashion to the wild type protein (Fig. 7), whereas Cav1Δ81–100-HA mainly targeted to the Golgi complex as judged by colocalization with the Golgi marker, GM130 (Fig. 7). GFP-dysf was largely localized to intracellular puncta in Cav1−/− cells (PM/IC ratio 0.67 ± 0.07) but PM association was restored by re-expression of either Cav1-HA (PM/IC ratio 1.99 ± 0.96) or Cav3-HA (PM/IC ratio 2.3 ± 0.55) (18) (Fig. 8, A and B). In contrast, expression of Cav1Δ81–100-HA (PM/IC ratio 0.61 ± 0.10), Cav3G55S-HA (PM/IC ratio 0.65 ± 0.15), or Cav3C71W-HA (PM/IC ratio 1.16 ± 0.57) did not affect dysferlin traffic to the PM and dysferlin remained enriched in intracellular vesicles of Cav1−/− MEFs (Fig. 8B). The lack of an inhibitory effect of these mutants on dysferlin endocytosis is also shown by the uptake of myc antibodies when these mutants are expressed together with GFP-dysf, as compared with the WT Cav3 protein (Fig. 8C).

The above results suggest that this conserved domain of caveolin is required for inhibition of dysferlin endocytosis and its retention at the PM.

Dominant acting mutants of Cav3 cause a reduction in surface Cav3, retention of Cav3 in the Golgi complex, and increased degradation (61, 62). We have previously shown that these mutants cause an accumulation of dysferlin in the Golgi complex. We investigated whether this was a result of a block of dysferlin exit from the Golgi rather than a consequence of redistribution due to dysferlin instability at the PM in the...
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**Exit from the Golgi Complex**—In cells devoid of caveolin dysferlin accumulates in intracellular vesicular structures, which we now show are endocytic in nature. No accumulation was observed in the Golgi complex and we demonstrated that despite the lack of surface labeling, dysferlin is rapidly transiting the cell surface in these cells, as shown by uptake of extracellular antibodies to a lumenal tag. These results suggest that dysferlin does not absolutely depend on caveolin for Golgi exit. Recent studies have suggested that novel exocytic carriers, containing defined quanta of caveolin, leave the Golgi complex and fuse directly with the PM (63). These carriers, termed exocytic caveolar carriers (64), form a novel exocytic pathway distinct from classical exocytic carriers (see scheme in Fig. 10); these carriers would presumably be absent in cells lacking caveolin. Our data provide new insights into these pathways. Full-length dysferlin can clearly utilize a non-caveolar carrier pathway to reach the PM, as shown in Cav1<sup>−/−</sup> cells. Similarly, a dysferlin mutant lacking all six C2 domains (Δ-C2) can also efficiently reach the PM, both in the absence or presence of caveolin again showing use of a non-caveolar carrier. However, in stark contrast to these two constructs, a protein of intermediate length, which lacks four C2 domains, shows an absolute dependence on caveolin for exit from the Golgi complex (Fig. 2 and scheme in Fig. 10). Whereas this is an artificially generated construct, these results clearly demonstrate a role for caveolin in Golgi exit, as suggested previously for a number of proteins including the angiotensin receptor (59), insulin receptor (65), and the stretch-activated channel, TRPC1 (66). The structural features that make these proteins dependent on caveolin for Golgi exit

**DISCUSSION**

In this work we have provided novel insights into dysferlin trafficking dynamics with respect to caveolin. Through the use of cells lacking caveolin, by expression of caveolin mutants, and by using different mutants of dysferlin, we have now identified the precise steps in dysferlin trafficking that are regulated by caveolin. We show directly that caveolins can facilitate exit of dysferlin mutants from the Golgi complex. In addition, dominant-acting caveolin mutants inhibit Golgi exit of mutant or wild type dysferlin. However, most unexpectedly, we now show that the primary effect of caveolin is to inhibit dysferlin endocytosis, implicating an endocytic mechanism in caveolin-associated muscle pathology.

**Ultrastructural characterization of dysferlin endocytosis in Cav1<sup>−/−</sup> and WT Cav1 MEFs.** WT Cav1 or Cav1<sup>−/−</sup> MEFs were transfected with GFPDysf and then after 14 h were incubated sequentially with antibodies to the lumenal myc tag and then HRP-labeled secondary (anti-mouse) antibodies at 4 °C. The cells were then warmed to 37 °C for 2 min to allow endocytosis to occur. The DAB reaction was performed on the living cells at 4 °C in the presence (+AA) or absence (−AA) of ascorbic acid as indicated. After fixation the transfected cells were identified under the light microscope by their GFP fluorescence and were marked to allow subsequent identification after embedding in resin. The marked areas were sectioned and viewed unstained. In the absence of AA, allowing visualization of both surface and intracellular pools of HRP, WT Cav1 cells showed HRP reaction product over the entire cell surface (A and B). In striking contrast, all transfected Cav1<sup>−/−</sup> cells showed sparse patchy surface labeling (C and D; arrowheads) consistent with greatly reduced retention of dysferlin at the plasma membrane. Quantitation of surface coverage by intersection counting (see “Experimental Procedures”) showed that 10.3% of the PM of Cav1<sup>−/−</sup> cells was covered in HRP reaction product and 89.0% of the PM of WT cells. Neighboring untransfected cells showed no trace of HRP labeling (results not shown) demonstrating the specificity of the antibody labeling. Potential clathrin-independent early endocytic carriers (arrowheads) were frequently observed in the Cav1<sup>−/−</sup> cells (E–G). In the presence of ascorbic acid to quench extracellular HRP, internal ring-shaped endocytic elements were clearly demonstrated in the Cav1<sup>−/−</sup> cells (H and I). Bars, A–D, 1 μm; E–I, 200 nm.
Evidence for dysferlin transport to the cell surface under these conditions suggesting that the caveolin mutants cause a complete block in Golgi exit. Interestingly, this was true for all the tested membrane-associated dysferlin constructs, including Δ-C2, which lacks most of the cytoplasmic domain and traffics to the PM in a caveolin-independent manner. This raises the possibility that mutant caveolin might perturb dysferlin trafficking at an earlier stage in the Golgi complex, before divergence of the two pathways. Consistent with this, Golgi caveolin mutants accumulate throughout the Golgi complex including the cis Golgi (18, 47). The specificity of this effect is shown by the fact that a Golgi-localized form of caveolin lacking amino acids 81–100 does not prevent dysferlin from exiting the Golgi. This suggests that a direct interaction between dysferlin and caveolin, at least, at the Golgi level may be taking place. If so, this narrows down the interacting domain of dysferlin to the TM domain and nearby cytoplasmic region, which contains four potential CSD binding motifs. However, these findings do not rule out perturbation of lipid domains of the Golgi complex, which may be influenced by expression of a form of caveolin with mutations in this potential lipid-binding domain (45). If so, these effects are restricted to specific cargo proteins as the transit of other proteins, such as GPI-anchored proteins, through the Golgi complex is unaffected by the expression of the mutant caveolin proteins (18).

**Caveolins Inhibit Dysferlin Endocytosis**—We show here for the first time that caveolins inhibit endocytosis of dysferlin. In cells lacking Cav1 and Cav3, dysferlin is rapidly cleared from the cell surface under these conditions suggesting that the caveolin mutants cause a complete block in Golgi exit. Interestingly, this was true for all the tested membrane-associated dysferlin constructs, including Δ-C2, which lacks most of the cytoplasmic domain and traffics to the PM in a caveolin-independent manner. This raises the possibility that mutant caveolin might perturb dysferlin trafficking at an earlier stage in the Golgi complex, before divergence of the two pathways. Consistent with this, Golgi caveolin mutants accumulate throughout the Golgi complex including the cis Golgi (18, 47). The specificity of this effect is shown by the fact that a Golgi-localized form of caveolin lacking amino acids 81–100 does not prevent dysferlin from exiting the Golgi. This suggests that a direct interaction between dysferlin and caveolin, at least, at the Golgi level may be taking place. If so, this narrows down the interacting domain of dysferlin to the TM domain and nearby cytoplasmic region, which contains four potential CSD binding motifs. However, these findings do not rule out perturbation of lipid domains of the Golgi complex, which may be influenced by expression of a form of caveolin with mutations in this potential lipid-binding domain (45). If so, these effects are restricted to specific cargo proteins as the transit of other proteins, such as GPI-anchored proteins, through the Golgi complex is unaffected by the expression of the mutant caveolin proteins (18).

**FIGURE 7.** Subcellular distribution of caveolin scaffolding domain mutants. Cav1−/− cells were transfected with HA-tagged Cav3, Cav3G55S, Cav3C71W, Cav1Δ81–100, and Cav3P104L, and colabeled with anti-HA and anti-GM130 antibodies. A, similarly to the wild type Cav3, the CSD mutants Cav3G55S and Cav3C71W CSD are targeted to the PM. In contrast, deletion of the CSD, Cav1Δ81–100, results in accumulation in the Golgi complex similarly to the dystrophy mutant Cav3P104L, demonstrated by the extensive overlap with the Golgi marker, GM130. B, CSD mutant predominant phenotypes were scored based on colabeling with relevant intracellular markers. Results are presented as percentage of cells showing a prevalent subcellular localization, and are representative of three individual experiments (n = 30–250 for each construct). Bar, 10 μm.
ultrastructural analysis of the endocytic pathway showing dysferlin in tubular/ring-shaped early endosomal elements. These results suggest a novel role of this pathway in regulating dysferlin surface expression and, a role for caveolin in inhibiting the clathrin-independent endocytosis of specific markers.

The inhibitory effect of caveolins on dysferlin endocytosis presents an interesting conundrum. We believe that a direct inhibitory effect of caveolin by binding dysferlin to immobile caveolar domains is unlikely; both electron microscopy and light microscopy show that dysferlin does not colocalize significantly with caveolin, even at the level of light microscopy, and both immuno-EM on frozen sections (18) and EM surface labeling experiments (this study) confirmed that dysferlin was not concentrated within caveolae. Thus, the inhibition by caveolin appears to be indirect. Yet our results suggest that the effect of caveolin on dysferlin endocytosis is specific; whereas acute Cav1 expression has been shown to inhibit clathrin-independent endocytosis of CTB (54, 70) and SV40 is efficiently internalized by cells devoid of caveolae (71) CTB and SV40 internalization was quantitatively identical in WT Cav1 and Cav1−/− MEFs as used here. Furthermore, no effect on GPI-AP internalization could be detected in Cav1−/− cells. This argues against a general negative inhibitory role of caveolins on clathrin-independent endocytosis, but suggests that caveolins specifically inhibit dysferlin entry into this pathway. We could also show that the increased uptake of dysferlin in Cav1−/− cells was not due to increased endocytosis caused by dysferlin expression (results not shown).

Caveolae have been suggested to be negative regulators of clathrin-independent endocytosis (70, 72) but other work identifies caveolae as endocytic vehicles (73–75). A new concept described here is that caveolin is regulating the non-caveolar endocytosis of dysferlin as we have
Caveolin Regulation of Dysferlin

A

B

FIGURE 9. Dystrophy mutant of Cav3 has a dominant inhibitory effect on dysferlin exit from the Golgi. Cav1Δ81–100 cells were co-transfected with GFP-Dysf and HA-tagged Cav3P104L or Cav1Δ81–100 and labeled with rabbit anti-HA (A and B) and mouse anti-GM130 (B) antibodies. Anti-myc antibody was internalized overnight (B). A, expression of Cav3P104L-HA blocks dysferlin exit from the Golgi apparatus. No significant Golgi pool of internalized myc antibodies is seen after overnight incubation at 37 °C. B, expression of Golgi-localized epitope-tagged Cav1Δ81–100 mutant does not affect GFP-dysf exit from the Golgi apparatus. Cav1Δ81–100, but not GFP-Dysf, is retained in the Golgi complex as demonstrated by colocalization with the Golgi marker, GM130. Bars, 10 μm.

FIGURE 10. Model for regulation of caveolin trafficking by caveolin. In WT Cav1 cells full-length dysferlin exit from the Golgi complex may take place via caveolar and noncaveolar exocytic carriers. In Cav1ΔΔ1 cells dysferlin must use an alternative pathway(s). However, a truncation mutant lacking part of the cytoplasmic domain (Δ-1) is completely dependent on caveolin for Golgi exit as it cannot enter the non-caveolar pathway. A more severe mutant (Δ-C2) traffics to the PM in a caveolin-independent manner as it lacks information for incorporation into caveolar carriers. Retention of dysferlin at the PM is dependent on caveolin/caveolae due to inhibition of dysferlin endocytosis by caveolin. A truncation of dysferlin lacking all six C2 domains, Δ-C2, is not internalized suggesting a role for the cytoplasmic domain in endocytosis.

clearly demonstrated that dysferlin is not concentrated in caveolae.

A model for the inhibitory effect of caveolin on endocytosis must take into account the intriguing finding that two Cav3 single point mutants, which occur naturally in the human population, did not inhibit dysferlin endocytosis in complete contrast to the wild type protein. This implicates the scaffolding domain of caveolin in its inhibitory activity. The two inhibitory mutants were initially reported as dystrophy mutants (11) but subsequently have been shown to occur as polymorphisms in the population (76, 77). Yet two previous studies have shown specific effects of these mutant proteins in cultured cells (50, 59). Further studies should elucidate the underlying mechanisms involved in the inhibitory activity of the wild type protein in comparison to these single point mutants and whether these mutations can contribute to disease under certain conditions.

In conclusion, these studies have elucidated distinct roles of caveolin in regulating dysferlin trafficking pathways, both in positively regulating exocytosis and negatively regulating endocytosis via a clathrin-independent pathway for which dysferlin acts as a new marker. An interesting possibility is that this inhibitory activity of caveolin on endocytosis is regulated in vivo, allowing modulation of the surface levels of dysferlin and membrane remodeling. Whether caveolin acts in a similar fashion on other surface proteins will require further investigation. The involvement of endocytosis in muscle disease may be a more general phenomenon. Another sarcolemmal protein, α-sarcoglycan, which is linked to a subset of muscle disease limb girdle muscular dystrophy 2D (78, 79), is translocated from the cell surface to endosomes upon perturbation of its PM stability (80). α-Sarcoglycan stability at the PM relies on a proper assembly of the sarcoglycan complex (80). Thus dysferlin and α-sarcoglycan represent examples of sarcolemmal proteins where endocytic mechanisms play a central role in maintaining the integrity of the PM.

These results provide new insights into the functions of caveolins and the mechanisms underlying caveolin-related diseases. In addition, they provide fundamental insights into the regulation of exocytic and endocytic trafficking pathways of membrane proteins in mammalian cells and the importance of this poorly characterized clathrin-independent endocytic pathway in surface remodeling of specific PM components.

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REFERENCES

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SUPPLEMENTARY MATERIAL

Figure S1. (A) Dysferlin is not localized to late endosomes in Cav1-/- MEFs. Cav1-/- MEFs expressing GFP-dysf were immunolabeled with markers of the late endosomal/lysosomal compartments. Dysferlin is targeted to an endosomal compartment that is distinct from late-endosomal/lysosomal structures. There is no evident colocalization with LBPA- or LAMP-1 positive compartments.

(B) Expression of Cav1 rescues dysferlin endocytic phenotype in Cav1-/- MEFs. WT Cav1 and Cav1-/- MEFs were co-transfected with GFP-dysf and Cav1-HA and anti-myc antibodies were internalized for 20 min at 37ºC. Cells were labeled with antibodies against endogenous caveolin or the HA tag. Dysferlin internalization is rescued to WT levels by expression of HA-tagged Cav1 in Cav1-/- MEFs. Very few punctate structures colabeled with GFP-dysf.

(C) WT and Cav1-/- MEFs have similar endocytic rates. WT Cav1 and Cav1-/- MEFs were allowed to internalise Tfn or CTB for 10min at 37ºC. There is no significant difference in the rate of endocytosis between WT and Cav1-/- MEFs as shown by quantification of the mean fluorescence intensity of internalized Tfn or CTB. Error bars are ± SD (n= 30). Bars, (A-B) 10µm.

Figure S2. Anti-myc IgGs and Fab fragments do not affect the endocytosis mechanism of dysferlin in MEFs. WT Cav1 (not shown) and Cav1-/- MEFs were transfected with GFP-dysf. IgGs or Fab fragments of anti-myc antibodies were cointernalized with Tfn for 40min at 37ºC. There was no difference in the extent of internalization between anti-myc IgGs and Fab fragments in WT (not shown) and Cav1-/- MEFs. Consistent with results shown in Figure 4, Cav1-/- MEFs internalize more IgGs or Fabs compared to WT Cav1 MEFs (not shown). Bars, 10µm.
Figure S1

A

**Cav1/−**

**GFP Dysf**

**Anti-LAMP1**

**GFP Dysf**

**Anti-LBPA**

B

**WT Cav1**

**Endogenous Cav1**

**Cav1/−**

**Endogenous Cav1**

**Cav1/−**

**Cav1-HA**

B

**GFP Dysf**

**Anti-Myc uptake**

C

![Histogram showing average pixel intensity](image)