Interaction abolition between mutant caveolin-1<sub>Δ62–100</sub> and ABCA1 reduces HDL-mediated cellular cholesterol efflux

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1. Introduction

The accelerated efflux of cholesterol is mediated by several prominent proteins [1–3]. Among these, caveolin-1, which has 178 amino acid residues and is the main structural component of caveolae, plays an important role in the regulation of lipid uptake and efflux [4–6]. Although it is an integral membrane protein, caveolin-1 lacks typical transmembrane domains [7]. Instead, its N- and C-terminal hydrophilic domains flank a 33-residue hydrophobic domain that is suggested to form a helical hairpin anchored in the cytoplasmic face of the lipid bilayer. The last 20 residues of the N-terminal hydrophilic domain are called the scaffolding domain (amino acids 81–101). This region is reported to be a domain that is responsible for its cellular function [8] via its interaction with different molecules such as PDGFRα/β, estrogen receptor, adenylyl cyclase/PLCβ2, PLD/PKCα, eNOS, and connexin 43 [9,10]. The region between amino acids 135 and 150 is a novel membrane-attachment domain [11]. The C-terminal domain can associate with membranes via 3 palmitate chains [12].

It is reported that the deletion of amino acids 1–60 at the N-terminal prevents the rear polarization of caveolin-1 and impedes caveola formation in primary embryonic fibroblasts (MEFs) [13]. The region between amino acids 62 and 100 is involved in oligomerization [14,15], which can be blocked if this region is impaired [16,17]. Caveolin-1 alone or in conjunction with several other factors can form large oligomers approximately 200–400 kDa in size [8,14,15]. The oligomerization of caveolin-1 is a critical step for its exit from the Golgi into the plasma membrane [18].

It has been demonstrated that ATP-binding cassette transporter A1 (ABCA1) affects cellular lipid efflux in the presence of HDL and apolipoprotein A-1 (apoA-1) [19]. In our previous study, we demonstrated the existence of interactions between ABCA1 and caveolin-1 and between ABCA1 and HDL, but not between HDL and caveolin-1 in cholesterol-loaded rat aortic endothelial cells (ECs) [1]. Moreover, HDL upregulates ABCA1 expression, which in turn modulates the oligomerization and exit of caveolin-1 from the Golgi to enhance cholesterol efflux in ECs [2]. ABCA1 knockdown results in the retention of caveolin-1 in the Golgi apparatus, disruption of its oligomerization, and attenuation of cholesterol efflux in ECs [20]. However, the functional domain of caveolin-1 that interacts with ABCA1 for cholesterol efflux remains unknown. In this study, we constructed a panel of mutant caveolin-1 proteins to further clarify the region of caveolin-1 that interacts with ABCA1 in ECs and human embryonic kidney (HEK) 293 cells. We also...
examined the effects of selected mutant caveolin-1 proteins on cellular HDL-mediated cholesterol efflux.

2. Materials and methods

2.1. Cell culture

Sprague–Dawley rats (4 weeks old) were euthanized by intraperitoneal injections of sodium pentobarbital (nembutal; 50 mg/kg body weight; Abbott Laboratories). Rings (1 mm thick) were cut from the thoracic aorta and cultured in Dulbecco’s minimum essential medium (DMEM; GIBCO, New York, NY, USA) supplemented with 10% fetal bovine serum (FBS) and penicillin–streptomycin (50 U/mL; Sigma, St. Louis, MO, USA) in a 5% CO₂/95% air atmosphere. To establish pure endothelial cell cultures, ring explants were removed after 3–4 days of culturing. Cultures exhibiting pure ECs that were maintained for 3–9 passages were used in the experiment [20]. HEK293 cells were kindly provided by Dr. CP Hu (Department of Life Science, Tunghai University, Taichung, Taiwan). We used HEK293 cells, which do not express caveolin-1, for comparison because ECs possess endogenous caveolin-1 [21]. The cells were cultured in T-25 flasks (Corning Glassworks, Corning, New York, NY, USA) at 37 °C in DMEM supplemented with 10% FBS and penicillin–streptomycin (50 U/mL) in a 5% CO₂/95% air atmosphere. The investigation conformed to the Guide for the Care and Use of Laboratory Animals published by the United States National Institutes of Health (NIH Publication No. 85-23, revised 1996), and all animal experiments were approved by the Animal Care and Utilization Committee of Tunghai University, Taiwan.

2.2. Transient transfection and cholesterol-loaded cells

The cells were grown in T-25 flasks or 6-well plastic dishes until they reached 70–80% confluence before inducing the expression of individual constructs by transfection with Lipofectamine™ 2000 (Invitrogen, CA, USA) according to the manufacturer’s instructions. In brief, at 24 h before transfection, 1 × 10⁵ cells were seeded per 6-well plate. On the day of transfection, 3 μg normal or mutant c-myc-tagged caveolin-1 and 3 μg FLAG-tagged ABCA1 were diluted in 250 μL serum-free DMEM. In a separate tube, 10 μL lipofectamine was diluted in 250 μL serum-free DMEM. The diluted DNA and lipofectamine were then gently mixed, and DMEM was added to the DNA/lipofectamine mixture. The final mixture was added to the cultured cells that were grown on the 6-well plates. After incubation at 37 °C for 5 h, 250 μL DMEM containing 20% serum was added to the cells, which were grown for an additional 24 h. The cholesterol-loaded cells were performed as described previously [20]. Briefly, subconfluent monolayers of endothelial or HEK293 cells were washed twice with PBS containing 2 mg/mL fatty acid-free albumin (FAPA) (Sigma) and incubated with DMEM containing 2 mg/mL FAPA and 50 μg/mL cholesterol in ethanol (10 mg/mL) for 48 h at 37 °C.

2.3. Construction of normal and mutant c-myc-tagged caveolin-1 and flag-tagged ABCA1 recombinant plasmids

The cDNA of the different caveolin-1 domain deletions were generated and amplified using the following primer sets: 5′-TCTCAGATATTCGCGCCCTTCAG-3′ and 5′-GGATCCCATCTTCTCTCAG-3′ for full-length caveolin-1, 5′-CAGCGATGCTGTCAGAATGCTG-3′ and 5′-CTCAGATATTCGCGCCCTTCAG-3′ for the deleted N-terminal domain of caveolin-1 (CA1), 5′-GATGCTTGGGTCGC-3′ and 5′-TGTGCTTGGGTCGC-3′ for the deleted oligomerization domain of caveolin-1 (CA2), 5′-GAAACGTGGTCTTCCTCCATGTCATC-3′ and 5′-TGTTGCTGTCA

CCATCTCCGATC-3′ for the deleted scaffolding domain of caveolin-1. The oligonucleotides were cloned into the EcoRI and XhoI restriction sites of the pcDNA 3.1-c-myc-tagged plasmid (Invitrogen). The FLAG-tagged ABCA1 were kindly provided by Dr. Michael Fitzgerald (Department of Medicine, Harvard Medical School, MA, USA). The sequences and orientations of the inserts were confirmed by auto-sequencing.

2.4. Immunoblot analysis

Cholesterol-loaded cells were grown in 6-well plastic dishes and incubated with DMEM containing 2 mg/mL FAPA or 2 mg/mL FAPA and HDL (50 g/mL; Intralcel) at 37°C for 0, 5, 15, or 30 min. To detect the oligomerized proteins, Dithiobis (DSP; Sigma) treatment was performed as described previously [3]. In brief, Dithiobis was dissolved in dimethyl sulfoxide (DMSO) immediately before use. The cells were then incubated with 250 μM of DSP solution for 1 h at room temperature. After incubation, the cells were washed three times with PBS and lysed with RIPA lysis buffer (Pierce, Rockford, IL, USA) with a Protease Inhibitor Cocktail Kit (Thermo, Rockford, IL, USA) at 4 °C for 30 min. The protein concentration was measured using a bicinchoninic acid (BCA) protein assay (Pierce), and the proteins were then separated by 4–15% gradient sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). After electrophoresis, the proteins were transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore, Bedford, MA, USA). Blots were probed with mouse anti-human c-myc antibody (1:3000, v/v) (Invitrogen) or rabbit anti-human caveolin-1 (1:5000, v/v) (Santa Cruz, CA, USA) or rabbit anti-FLAG antibody (1:1000, v/v) (ABCam, Cambridge, MA, USA) followed by HRP-conjugated goat anti-mouse IgG (1:3000 or 1:5000, v/v) (Zymed, CA, USA). After washing with PBS containing 0.5% Tween 20, peroxidase activity was assessed using enhanced chemiluminescence (PerkinElmer Life Science, Boston, MA, USA).

2.5. Co-immunoprecipitation of caveolin-1 and ABCA1 in cholesterol-loaded cells after HDL incubation

The co-immunoprecipitation assay was performed as described previously with minor modifications [20]. In brief, cholesterol-loaded transfected cells were grown in 6-well plastic dishes and incubated with DMEM containing 2 mg/mL FAPA or both 2 mg/mL FAPA and HDL (50 g/mL; Intracel) at 37 °C for 5 min. The cells were washed twice with PBS and then incubated with DSP (250 μM; Sigma) at room temperature for 1 h. After incubation, the cells were washed three times with PBS and lysed with RIPA lysis buffer (Pierce) with a Protease Inhibitor Cocktail Kit (Thermo) on ice for 30 min. The cell lysates (500 μg) were incubated with anti-FLAG agarose (Sigma) on a rocking platform overnight at 4 °C. After incubation, the lysate and anti-FLAG agarose mixtures were centrifuged at 8200g for 3 min at 4 °C and subsequently washed three times with TBS containing 0.1% Triton X-100 (Sigma). The mixture was centrifuged at 4500g for 3 min at 4 °C, and the resultant pellets were harvested and incubated with 3 × FLAG peptides (150 ng/mL; Sigma) for 30 min at 4 °C. After incubation, the mixture was centrifuged again at 8200g for 3 min at 4 °C, and the supernatants were harvested and analyzed by immunoblotting. Mouse anti-human c-myc antibody (1:3000, v/v) (Invitrogen) or rabbit anti-FLAG antibody (1:1000, v/v; Abcam) was used in the immunoblot analysis.
2.6. Immunofluorescence staining

Cholesterol-loaded cells grown on a cover slip in 6-well plates for 24 h were washed three times with PBS-albumin followed by incubation with DMEM containing 50 μg/mL HDL and 2 mg/mL FAFA at 37 °C for 5 min. The cells were then chilled on ice, washed three times with cold PBS-albumin, and fixed in 2% paraformaldehyde for 30 min on ice. After fixation, the cells were blocked with PBS containing 3% FBS for 30 min at room temperature. After the cells were washed with PBS, the cover slip cultures were incubated with mouse anti-human c-myc (1:250, v/v; Invitrogen) or rabbit anti-FLAG (1:200, v/v; Abcam) for 1 h at room temperature. The cover slips were washed three times with PBS and incubated for 30 min with TRITC-conjugated goat anti-mouse IgG (1:500, v/v; Zymed) or FITC-conjugated goat anti-rabbit IgG (1:500, v/v). The cover slips were washed three times with PBS, mounted on slides, and visualized using an LSM 510 confocal microscope (Zeiss, Germany).

2.7. Cholesterol efflux

The cholesterol efflux assay was performed as described previously with minor modifications [20]. The cells grown in 6-well plates were incubated with DMEM containing 2 mg/mL FAFA and

![Diagram of caveolin-1 mutants](https://example.com/diagram.png)

**Fig. 1.** Diagram and immunoblotting analysis of various caveolin-1 mutants used in this study. (A) The construction of the full-length and the five deletion mutants are shown. (B) ECs and HEK293 cells were transfected with various full-length or caveolin-1 deletion mutants for 24 h. Immunoblotting shows the expression levels of caveolin-1. LacZ is a c-myc-tagged control construct.
2.8. Statistical analysis

Data are expressed as means ± SD. Treatment groups were compared using two-tailed t-tests with SAS or ANOVA software. P < 0.05 was considered statistically significant.

3. Results

3.1. Cellular expression of various mutant caveolin-1 deletion constructs

To verify which domain of caveolin-1 interacts with ABCA1, we created various constructs containing caveolin-1 domain deletion mutants (Fig. 1A). The results of immunoblotting showed that the various forms of caveolin-1 mutants are expressed in the cells after transfection (Fig. 1B).

3.2. Mutant caveolin-1Δ62–100 does not interact with ABCA1 in cholesterol-loaded cells

We analyzed the interaction between wild-type and various forms of mutant caveolin-1 with ABCA1 by immunoprecipitation. The results indicate that ABCA1 interacts with full-length caveolin-1, mutant caveolin-1Δ135–178, caveolin-1Δ102–133, caveolin-1Δ83–100, and caveolin-1Δ1–60, but not caveolin-1Δ62–100 or the oligomerization domain of caveolin-1 in cholesterol-loaded rat aortic ECs (Fig. 2A). Furthermore, the oligomerization domain of caveolin-1Δ62–100 alone abolished its interaction with ABCA1 in HEK293 cells (Fig. 2B). Taken together, the results suggest that the oligomerization domain of caveolin-1Δ62–100 is important for its interaction with ABCA1 and that this event is not cell-type specific.

3.3. ABCA1 colocalizes with full-length caveolin-1 but not mutant caveolin-1Δ62–100

To further confirm the colocalization of ABCA1 with mutant caveolin-1Δ62–100 in cholesterol-loaded rat aortic ECs after HDL incubation, immunofluorescence confocal microscopy was performed. The results show that full-length caveolin-1 colocalized with ABCA1 in the cholesterol-loaded ECs after HDL incubation (Fig. 3A–C). However, mutant caveolin-1Δ62–100 did not colocalize with ABCA1 (Fig. 3D–F). Similarly, the colocalization of full-length caveolin-1 and ABCA1 occurred in cholesterol-loaded HEK293 cells (Fig. 3G–I). Mutant caveolin-1Δ62–100 also did not colocalize with ABCA1 in cholesterol-loaded HEK293 cells after HDL incubation (Fig. 3J–L).

3.4. Mutant caveolin-1Δ62–100 does not interact with ABCA1 after HDL incubation

Our previous study indicates that cholesterol efflux occurs rapidly after HDL incubation [2]. The immunoblotting results show that caveolin-1 and ABCA1 interact after 5-min HDL incubation (Supplemental Fig. 1). However, the interaction between the oligomerization domain of caveolin-1Δ62–100 and ABCA1 was not detected after HDL incubation (Supplemental Fig. 1).

3.5. Mutant caveolin-1Δ62–100 blocks oligomerization after HDL incubation

HDL incubation enhanced the oligomerization of full-length caveolin-1 but not that of mutant caveolin-1Δ62–100. Our previous study indicates that HDL incubation enhances caveolin-1 oligomerization [2]. In the present study, we aimed to verify the effect of HDL incubation on mutant caveolin-1Δ62–100 oligomerization. The results show that the oligomerization of full-length caveolin-1 increased after 15 min HDL incubation. However, there were no detectable oligomer forms of mutant caveolin-1Δ62–100 (Supplemental Fig. 2). This finding suggests that HDL incubation only enhances the oligomerization of full-length caveolin-1 and not of mutant caveolin-1Δ62–100.

3.6. Mutant caveolin-1Δ62–100 impairs cellular cholesterol efflux

Our previous study indicates that the overexpression of full-length caveolin-1 enhances cholesterol efflux in ECs after HDL incubation [2]. The results of the present study indicate that the cholesterol efflux in full-length caveolin-1 and ABCA1-co-transfected cells is greater than that in mutant caveolin-1Δ62–100 and ABCA1-co-transfected cells (Fig. 4). In other words, mutant caveolin-1Δ62–100 reduces cholesterol efflux in both ECs and HEK293 cells (Fig. 4).

4. Discussion

The oligomerization of caveolin-1 was first discovered using velocity gradient centrifugation [14,15]. The physiological roles of caveolin-1 oligomerization are still unclear except those regarding the formation of caveolae structures and the exit of caveolin-1 from the Golgi [10]. In this study, several lines of evidence eluci-

Fig. 2. Mutant caveolin-1Δ62–100 abolishes the interaction with ABCA1 in cholesterol-loaded cells after HDL incubation. After co-transfection with caveolin-1 and ABCA1, ECs (A) and HEK293 cells (B) were incubated with cholesterol (50 μg/ml) for 48 h followed by treatment with HDL (50 μg/ml) for 15 min. The cells were washed three times with PBS and exposed to DSP cross-linker for 1 h. Total cell lysates were immunoprecipitated with anti-FLAG antibody and were subjected to immunoblot analysis.
date the physiological roles of caveolin-1 oligomerization. First, the deletion of the caveolin-1 oligomerization domain abolishes the colocalization and interaction between caveolin-1 and ABCA1. Second, HDL incubation only enhances the oligomerization of caveolin-1 and not that of caveolin-1\(\Delta62-100\). Finally, the deletion of the caveolin-1 oligomerization domain results in a loss of function of the regulation of HDL-mediated cholesterol efflux. Considering the evidence, we believe that caveolin-1-mediated cholesterol efflux is dependent on the interaction between ABCA1 and caveolin-1 via its oligomerization domain.

In our previous study, we found that oligomerized full-length caveolin-1 interacts with ABCA1. However, the domain by which caveolin-1 binds to ABCA1 is still unknown. Orso et al. [22] demonstrated that caveolin-1 is retained in the Golgi apparatus in Tangier disease fibroblasts, which express various point mutation-containing ABCA1s. We demonstrated that ABCA1 interacts with caveolin-1; it also modulates caveolin-1 oligomerization, which is required for the Golgi exit of caveolin-1 [2]. This raises the possibility that the point mutants of ABCA1 in Tangier disease are important for its interaction with caveolin-1. However, the interaction between caveolin-1 and ABCA1 mutants in Tangier disease fibroblasts needs to be clarified in the future.

Besides ABCA1, the caveolin-1 scaffold domain within the oligomerization domain is important for its interaction with several proteins such as PDGFR\(\alpha/\beta\) [23], estrogen receptor [24], adenylyl cyclase/PLC\(\beta2\) [25], PLD/PKC\(\alpha\) [26], eNOS [27], and connexin 43 [28]. These interacting proteins are located in the caveola structure and are important in the caveolin-1-mediated signaling pathway [10]. However, most of these interactions depend on lipid–protein interactions rather than protein–protein interactions [10]. An in vitro pull-down assay shows that purified ABCA1 directly interacts with purified caveolin-1, indicating that the interaction between ABCA1 and caveolin-1 is a protein–protein interaction (unpublished data). Determining the role that ABCA1 plays in these different signaling pathways may shed light on the underlying mechanism of caveolin-1-dependent signaling pathways.

Fig. 3. ABCA1 colocalizes with full-length caveolin-1 but not with mutant caveolin-1\(\Delta62-100\). Immunofluorescence micrographs show the distribution of caveolin-1 (red fluorescence) (A and C) and ABCA1 (green fluorescence) (B and H). Full-length caveolin-1 colocalized with ABCA1 in ECs (C) and HEK293 cells (I). Immunofluorescence micrographs show the distribution of mutant caveolin-1\(\Delta62-100\) (red fluorescence) (D and J) and ABCA1 (green fluorescence) (E and K). The results indicate that mutant caveolin-1\(\Delta62-100\) does not colocalize with ABCA1 in ECs or HEK293 cells (F and L). Scale bar: 5 \(\mu\)m (for interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).
Many physiological functions, such as accelerated cholesterol efflux, are regulated by numerous protein–protein interactions in various types of cells [10]. Full-length caveolin-1 and ABCA1 are colocalized in cholesterol-loaded aortic ECs after HDL incubation [120]. We also suggest that the molecular interaction between caveolin-1 and ABCA1 is associated with HDL-mediated cholesterol efflux via the transportation of cholesterol from the Golgi to the plasma membrane [20]. Our previous results also show that ABCA1-knockdown results in the retention of caveolin-1 in the Golgi apparatus and the disruption of its oligomerization. We suggest that ABCA1 modulates caveolin-1 transport from the Golgi to the plasma membrane by elevating caveolin-1 oligomerization during HDL-mediated cholesterol efflux [2,20]. It is reported that the region of caveolin-1 between amino acids 62 and 100 is involved in an oligomerization step [14,15] that is critical for its exit from the Golgi to the plasma membrane [18]. Lin et al. [2] also suggest that ABCA1 modulates the oligomerization and exit of caveolin-1 from the Golgi during HDL-mediated cholesterol efflux in aortic ECs. It is reported that deletions in caveolin-1 between amino acids 60 and 100 result in the retention of caveolin-1 in the ER [17]. However, the functional domain of caveolin-1 that interacts with ABCA1 for cholesterol efflux is not entirely clear. Herein, the results of immunofluorescence confocal microscopy and co-immunoprecipitation show that mutant caveolin-1 Δ62–100 neither colocalizes nor interacts with ABCA1 in cholesterol-loaded rat ECs or HEK293 cells after HDL incubation, respectively (Figs. 2 and 3 and Supplemental Fig. 1). To the best of our knowledge, the proteins associated with caveolin-1 that are involved in its oligomerization and cholesterol efflux remain unclear [10]. In this study, HDL incubation enhanced the oligomerization of full-length caveolin-1 but not that of mutant caveolin-1 Δ62–101 (Supplemental Fig. 2). Concomitantly, cholesterol efflux was significantly reduced in mutant caveolin-1 Δ62–100 cells after HDL incubation for 5, 15, and 30 min in ECs and HEK293 cells (Fig. 4A and B). The data show that cholesterol efflux did not significantly increase 30 min after HDL incubation in ECs overexpressing full-length caveolin-1 (Fig. 4A); this might be attributable to the abundance of endogenous caveolin-1 in ECs [2]. These results provide strong evidence that the oligomerization domain of caveolin-1 is important in the interaction between caveolin-1 and ABCA1 as well as in HDL-mediated cholesterol efflux in primary cells and cell lines.

Conflict of interest
None declared.

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Appendix A. Supplementary data

References


