Biochemical and Biophysical Research Communications 379 (2009) 460–465

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Flotillin-1 stabilizes caveolin-1 in intestinal epithelial cells

Elena V. Vassilieva [a], Andrei I. Ivanov [b], Asma Nusrat [a,*]

[a] Epithelial Pathobiology Research Unit, Department of Pathology and Laboratory Medicine, Emory University, Whitehead Research Building, 615 Michael Street, Atlanta, Georgia 30322, United States
[b] Division of Gastroenterology and Hepatology, Department of Medicine, University of Rochester, Rochester, NY 14642, United States

ARTICLE INFO

Article history:
Received 3 December 2008
Available online 31 December 2008

Keywords:
Epithelial cells
Lipid rafts
Lactosyl ceramide internalization
Lysosomal degradation

ABSTRACT

Flotillins and caveolins represent two types of resident proteins associated with lipid rafts in mammalian cells, however, their possible cross-talk in regulating lipid raft functions remains poorly understood. In this report, we observed that siRNA-mediated down-regulation of flotillin-1 expression which disrupted lipid raft-mediated endocytosis of BODIPY FL C_5-lactosylceramide also substantially decreased caveolin-1 level in SK-CO15 human intestinal epithelial cells. The decrease in caveolin-1 expression appeared to be specific for flotillin-1 knock-down and was not observed after down-regulation of flotillin-2. The decrease in caveolin-1 level in flotillin-1-depleted cells was not due to suppression of its mRNA synthesis and was not mimicked by cholesterol depletion of SK-CO15 cells. Furthermore, flotillin-1 dependent down-regulation of caveolin-1 was reversed after cell exposure to lysosomal inhibitor, chloroquine but not proteosomal inhibitor, MG262. Our data suggest that flotillin-1 regulates caveolin-1 level by preventing its lysosomal degradation in intestinal epithelial cells.

The lipid rafts represent specialized dynamic areas (domains) of eukaryotic membranes which are characterized by unique chemical composition and physical properties [1,2]. The most vivid feature of lipid raft is their enrichment in particular lipids such as cholesterol and sphingolipids, which create a microenvironment for recruitment of various membrane and cytosolic proteins with high binding affinity to these lipids. By clustering/segregating different lipid and protein constituents of the membrane, rafts mediate many crucial cellular functions, including regulation of signaling by plasma membrane receptors, activity of ion pumps and channels and mediation of vesicle trafficking/fusion [3,4]. Although membrane rafts are readily formed in a pure lipid environments of liposomes, in living cells these lipid domains are frequently supported by specialized proteins which are important modulators of raft structure, stability and functions [5]. Resident lipid raft proteins flotillins and caveolins are co-expressed in many types of mammalian cells, and as such they are frequently enriched in the same cell membranes. However, several recent studies have demonstrated that flotillins and caveolins do not intermix and populate distinct subsets of lipid rafts [6–10].

The flotillin (also known as the reggie) protein family consists of two ubiquitously expressed isoforms, flotillin-1 and flotillin-2. Flotillins are evolutionary highly conserved proteins [11] that belong to the SPFH (for stomatins, prohibitins, flotillins, HflK/C) protein superfamily and have propensity for oligomerization [12,13]. Flotillins are enriched at the plasma membrane where they associate with the inner leaflet via hydrophobic amino acid stretches and posttranslationally attached acyl groups of palmitic and myristic acids [13–15]. At the plasma membrane, flotillin-1 and flotillin-2 are organized as homo- and hetero-tetramers and flotillin-2 is required for stabilization of flotillin-1 [12,16]. Flotillins readily induce membrane curvature and reportedly mediate a unique clathrin-independent endocytic pathway [6,9]. Beside their proposed role in endocytosis, flotillins are shown to participate in signaling by GPI-anchored plasma membrane proteins and in regulation of the actin cytoskeleton reorganizations, and actin-dependent cell adhesion and motility [15,11].

Caveolins are well-characterized resident lipid raft proteins, which participate in the formation of a distinct subset of the membrane rafts called caveolae [17–19]. Three isoforms of caveolin are known: ubiquitously expressed caveolin-1 and caveolin-2, and muscle-specific caveolin-3. Knock-down experiments in cultured cells and in vivo in genetically modified mice yielded important information about role of caveolins in caveolae formation at the plasma membrane and caveolar endocytosis [20]. For example, downregulation of caveolin-1 impaired albumin uptake in bovine aortic endothelial cells [21] and inhibited internalization of low-density lipoprotein receptor-related protein 6 in HeLa cells [22]. Similarly to flotillins, caveolin isoforms undergo homo- and hetero-association at the cell membranes. Such inter-isoform associations may stabilize caveolins in the cells since expression of caveolin-2 was shown to be strongly downregulated in caveolin-1 knock-down mice, possibly by accelerating its degradation, [20].

* Corresponding author. Fax: +1 404 727 3321.
E-mail address: anusrat@emory.edu (A. Nusrat).

0006-291X/$ - see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2008.12.118

Although flotillins and caveolins occupy topographically distinct domains of the plasma membrane and do not physically associate [23,24,10,9], they may cooperate functionally by controlling different steps of protein endocytosis [25,26]. Therefore, it is important to know if these two types of lipid raft modulators cross-talk and influence each other expression and functions. Recent studies have demonstrated that cellular levels and localization of flotillin-1 and flotillin-2 do not depend on caveolin-1 expression in fibroblasts and epithelial cells [8,10]. However, whether flotillins regulate expression of caveolins has not been investigated. Here, we report a serendipitous observation that selective down-regulation of flotillin-1 expression in SK-CO15 human intestinal epithelial cells caused dramatic decrease in protein level of caveolin-1. Such decrease in caveolin-1 expression was not due to diminished mRNA level, but was a consequence of accelerated lysosomal degradation of this protein. These data provide the first evidence of an interplay between flotillins and caveolins and suggest that flotillin stabilizes caveolin-1 in living cells and therefore my regulate caveolin-1 functions.

Materials and methods

Antibodies and other reagents. The following primary polyclonal (pAb) and monoclonal (mAb) antibodies were used for immunoblotting: flotillin-1 mAb clone 18, flotillin-2 mAb clone 29, caveolin-1 pAb (BD Biosciences, San Jose, CA); α-actin pAb (Sigma–Aldrich, St. Louis, MO). Horseradish peroxidase-conjugated goat anti-rabbit and anti-mouse secondary antibodies were obtained from Jackson Immunoresearch Laboratories (West Grove, PA). BODIPY FL C_5-lactosylceramide (BODIPY-LacCer) was obtained from Invitrogen (Carlsbad, CA), MG262 was purchased from Biomol Research Laboratories (Plymouth Meetings, PA), chloroquine, methyl-β-cyclodextrin and other reagents were obtained from Sigma.

Cell culture and RNA interference. SK-CO15 cells were provided by Dr. E. Rodriguez-Boulan, Weill Medical College of Cornell University, NY and were cultured in DMEM as described previously [27–29]. Cells were grown on 6-well plates or on collagen-coated cover slips for immunoblotting and immunofluorescence labeling respectively. RNA interference experiments were performed using siRNA SmartPools for human flotillin-1, flotillin-2, caveolin-1, and lamin A/C as control (Dharmacon, Lafayette, CO). SK-CO15 cells were plated at 60–70% confluency and were transfected next day with 50–100 nm of siRNA using DharmaFect 1 transfection reagent (Dharmacon) according to a manufacturer protocol. Cells were analyzed 72–98 h post-transfection. For the inhibitory analysis, transfected SK-CO15 cells were incubated with either 0.1 mM chloroquine or 10 nm MG262 for 22 h in DMEM. Cholesterol depletion was performed by treating cells with 5 mM methyl-β-cyclodextrin for 23 h in a serum-free DMEM.

Real-time RT-PCR. Total RNA was isolated from SK-CO15 cells using TRIsol LS reagent (Invitrogen) according to the manufacturer instruction. RNA samples treated with DNAseI (Invitrogen) to remove genomic DNA. One-step quantitative real-time RT-PCR was performed using BioRad iCycler and iScript One-step PT-PCR kit containing a SYBR Green dye (BioRad, Hercules, CA). Sequences for caveolin-1 and a housekeeping control 18S rRNA primers were published elsewhere [30,31], primer oligonucleotides were synthesized by IDT (Coralville, IA).

Immunoblotting. Cells were homogenized in Laemmli sample buffer containing a proteinase inhibitor cocktail (1:100, Sigma) and phosphatase inhibitor cocktails 1 and 2 (both at 1:200, Sigma). Lysates were supplemented with β-mercaptoethanol and boiled. SDS polyacrylamide gel electrophoresis and immunoblotting were conducted by standard protocols with 10–20 μg protein per lane. Proteins of interest were visualized after their transfer on nitrocellulose membrane using appropriate primary and HRP-conjugated secondary antibodies. Results shown are representative immunoblots of at least three independent experiments. Protein expression was quantified by densitometric analysis of immunoblot images using UN-SCAN-IT digitizing software (Silk Scientific, Orem, UT).

BODIPY-LacCer internalization assay. SK-CO15 cells were transfected with flotillin or control siRNAs, replated on coverslips 54 h post-transfection, and 18 h later were subjected to internalization assay. The assay was modified from protocol described elsewhere [32]. Briefly, SK-CO15 cells were washed in serum-free DMEM and incubated with 1 μM BODIPY-LacCer in the same media at for 30 min at 4 °C. Unbound BODIPY-LacCer was removed by washing cells with cold DMEM and internalization was started by placing cells at 37 °C for 5 min. Internalization was stopped by transferring cells back on ice and non-internalized BODIPY-LacCer was removed from the cell surface by incubating cells in the same medium supplemented with 5% fatty-acid free BSA for 1 h on ice, exchanging the medium for fresh every 10 min. Cells were fixed with 3.7% paraformaldehyde and were mounted on slides using ProLong Antifade medium (Invitrogen). The slides were examined using a Zeiss LSM510 laser scanning confocal microscope (Zeiss Microimaging Inc., Thornwood, NY) coupled to a Zeiss 100M axiovert and 63× or 100× Pan-Apochromat oil lenses. Image analysis including pixel intensity measurement was performed using Axiovision software (Release 4.3, Zeiss).

Results and discussion

To examine possible relationships between two types of resident lipid raft proteins in intestinal epithelial cells, expression of flotillin isoforms and caveolin-1 was down-regulated using RNA interference in a SK-CO15 colonic epithelial cell line. Fig. 1A and B shows that transfection of SK-CO15 cells with flotillin-1 or flotillin-2-specific siRNA SmartPools resulted in approximately 94% and 95% decrease in expression of targeted proteins, respectively. Furthermore, knock-down of either flotillin isoform mutually down-regulated each other levels (Fig. 1A and B). This finding is consistent with previous report [33] and is likely to reflects known hetero-oligomerization of flotillin-1 and flotillin-2 which stabilizes them at cell membranes [16,12]. More interestingly, we observed that downregulation of flotillin-1 induced a substantial (∼65%) decrease in the level of caveolin-1, whereas flotillin-2 knock-down did not affect caveolin-1 expression (Fig. 1A and B). Such a selective effect of flotillin-1 knock-down on caveolin-1 expression is surprising since we observed significant down-regulation of flotillin-1 in flotillin-2-depleted cells (Fig. 1A and B). However, this observation can be explained by the different extent of flotillin-1 down-regulation. Indeed only ∼6% of initial flotillin-1 remained in SK-CO15 cells treated with flotillin-1-specific siRNA, whereas approximately 20% of this isoform was detected in flotillin-2-depleted cells (Fig. 1B). Since flotillin-1 is an abundant intracellular protein, even 20% of its normal level may be sufficient in mediating some of its cellular activities and a significant depletion is required to demonstrate the entire functional effects of flotillin-1 knock-down. On the other hand, siRNA-mediated downregulation of caveolin-1 did not affect flotillin-1 or flotillin-2 protein level (Fig. 1C), which indicates a lack of mutual regulation of these two types of lipid raft proteins. To our best knowledge, this is the first report that flotillin-1 cross-talks with caveolin-1 by regulating its expression. Our findings are also in line with two recent studies of caveolin-1 null and caveolin-1 expressing fibroblasts and thyroid epithelial cells, which showed that cellular level and localization of flotillin-1 is regulated in caveolin-1-independent fashion [8,10].