Possible regulation of caveolar endocytosis and flattening by phosphorylation of F-BAR domain protein PACSIN2/Syndapin II

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ABSTRACT. Caveolae are flask-shaped invaginations of the plasma membrane. The BAR domain proteins form crescent-shaped dimers, and their oligomeric filaments are considered to form spirals at the necks of invaginations, such as clathrin-coated pits and caveolae. PACSIN2/Syndapin II is one of the BAR domain-containing proteins, and is localized at the necks of caveolae. PACSIN2 is thought to function in the scission and stabilization of caveolae, through binding to dynamin-2 and EHD2, respectively. These two functions are considered to be switched by PACSIN2 phosphorylation by protein kinase C (PKC) upon hypotonic stress and sheer stress. The phosphorylation decreases the membrane binding affinity of PACSIN2, leading to its removal from caveolae. The removal of the putative oligomeric spiral of PACSIN2 from caveolar membrane invaginations could lead to the deformation of caveolae. Indeed, PACSIN2 removal from caveolae is accompanied by the recruitment of dynamin-2, suggesting that the removal provides space for the function of dynamin-2. Otherwise, the removal of PACSIN2 decreases the stability of caveolae, which could result in the flattening of caveolae. In contrast, an increase in the amount of EHD2 restored caveolar stability. Therefore, PACSIN2 at caveolae stabilizes caveolae, but its removal by phosphorylation could induce both caveolar endocytosis and flattening.

KEYWORDS. BAR domain, caveolae, mechanical stress, phosphorylation, protein kinase C

ABBREVIATIONS. BAR, Bin/Amphiphysin/Rvs; PKC, protein kinase C; PRD, proline-rich domain; TIRFM, total internal reflection fluorescence microscopy

INTRODUCTION

Caveolae are flask-shaped invaginations of the plasma membrane, with diameters from 50 to 100 nm. Caveolae are present in various cell types and have been implicated in many cellular processes, such as endocytosis and signal transduction.¹ Initially, caveolae were thought
to function as endocytic organelles that internalize extracellular materials or membrane. However, caveolae are immobile signaling platforms anchored to the actin cytoskeleton. Caveolin, the major structural protein in caveolae, is an evolutionarily conserved integral membrane protein. Caveolae contain many cellular components, such as sphingolipids, GM1 gangliosides, cholesterol, caveolin-1–3, cavin1–4, protein kinase C (PKC) α, EHD2, the F-actin cross-linking protein filamin, the promoter of actin-filament elongation mDia1, the Bin/Amphiphysin/Rvs (BAR) domain-containing proteins (BAR proteins, described below) and others. Oligomerization of caveolin with cavins promotes the formation and maintenance of caveolae, by the generation of caveolar coats. Caveolar endocytosis is activated by PKCα, while scission from the plasma membrane is thought to be mediated by the ubiquitously expressed protein dynamin-2, a mechanochemical GTPase that oligomerizes at the necks of caveolae.

In the resting state, caveolin-1 exhibits slow turnover in the plasma membrane, suggesting a tightly packed caveolar structure. However, under hypotonic conditions and during the activation of kinases such as PKC or the disruption of the actin cytoskeleton, caveolin-1 becomes relatively more mobile, indicating the dynamic regulation of caveolar molecules under such conditions. Therefore, it was suggested that caveolae function as mechanosensors by responding to membrane tension under regulation of protein kinases.

When cells are exposed to shear stress, caveolae formation is observed. Furthermore, caveolae act as membrane reservoirs in response to membrane tension under hypotonic conditions; i.e., they function as a buffer that unfolds upon membrane tension. The unfolding of the concave membrane of caveolae results in their flattening, which can increase the cellular surface area. During this process, the caveolar components, such as caveolin-1 and glycosphingolipids, are redistributed. These results indicate that caveolae play an important role in mechanotransduction, which might be related to the onset and progression of vascular proliferative disease. Furthermore, caveolae have been proposed to play crucial physiological roles in tumorigenesis, muscular disorders, cardiomyopathy, and other diseases, which might be dependent on the mechanical stress applied to cells. Hence, it is important to understand the mechanisms underlying the regulation of caveolae.

The BAR proteins involved in caveolae

The BAR domains are evolutionarily conserved protein domains. The BAR domains form crescent-shaped homo-dimers, which sense and/or generate membrane curvature through binding to the membrane. The BAR domains have positively charged surfaces, which are considered to function as templates for membrane curvature. The BAR domains polymerize into helical coats or oligomeric spirals, through lateral and tip-to-tip interactions, to deform the membrane into tubules, and these properties are thought to be important for the determination of membrane shape (Fig. 1). The BAR domain superfamily consists of 3 subfamilies: the BAR domain, the F-BAR/EFC domain, and the I-BAR/IMD domain. Caveolin is associated with the F-BAR domain proteins PACSIN2/Syndapin II and Nostrin. The F-BAR domain protein PACSIN2 regulates the morphogenesis and endocytosis of caveolae, through the positively charged concave surface that binds to membranes. The role of Nostrin in caveolar biogenesis has not been clarified yet.

The PACSIN2 binding proteins connect to actin filaments

Caveolin-1 is tethered to the cortical actin cytoskeleton via filamin. However, PACSIN2 and the binding proteins also provide the connection to actin filaments. The BAR
domain-containing proteins typically have SH3 domains. PACSIN2 has the F-BAR domain and the SH3 domain (Fig. 1). The C-terminal SH3 domain of PACSIN2 associates with the proline-rich domain (PRD) of dynamin-2 and N-WASP, that promotes membrane scission and activates the Arp2/3 complex-mediated actin polymerization, respectively. Nostrin binds to N-WASP through its SH3 domain. Although PACSIN and Nostrin bind to N-WASP, the roles of N-WASP and the Arp2/3 complex in caveolar functions remain unclear. Instead, mDia1 is reported to regulate actin polymerization in caveolae. However, the binding of mDia1 to PACSIN2 has not been examined.

Besides the SH3 domain, the Asn-Pro-Phe (NPF) sequence, in the linker between the F-BAR and SH3-domains of PACSIN2, binds to the Eps15 homology domain of the dynamin-like EHD2 ATPase. EHD2 binds to cavin1 and is localized to the necks of caveolae, where it stabilizes and constrains caveolae at the plasma membrane. Nucleotide hydrolysis by EHD2 is slower than that by dynamin, thus, EHD2 may control the slow dynamics of caveolae, which are considered to be important for the stabilization of caveolae. EHD2 exists at stationary caveolae and dissociates from caveolae after caveolar endocytosis. EHD2 constrains the lateral movement of caveolae, by linking the caveolae to actin filaments. Consistently, the depletion of EHD2 results in the mobilization of caveolae. Therefore, PACSIN2 and the PACSIN2-EHD2 complex play key roles in stabilizing caveolae, by associating with actin filaments.

There are several other binding proteins of PACSIN2, which include endosomal protein MICAL-L1, cation channel TRPV4, phosphoinositide phosphatase Synaptojanin-1, the Ras/Rac guanine nucleotide exchange factor Sos, and small GTPase Rac1. However, the roles of these proteins in caveolae have been not well understood.
FIGURE 2. A model of caveolar endocytosis mediated by PACSIN2 phosphorylation through PKC activation by mechanical stress. PACSIN2 oligomerizes and binds to the necks of caveolae. PACSIN2 is phosphorylated by PKC upon mechanical stimuli, such as hypotonic stress and shear stress, and phosphorylated PACSIN2 dissociates from the necks of caveolae. This enables dynamin-2 to occupy the space after the removal of PACSIN2, which induces caveolar scission and endocytosis. Otherwise, the loss of PACSIN2 at the caveolar neck leads to caveolar flattening, to buffer membrane tension.
Interestingly, the F-BAR domain of PACSIN2 was shown to directly bind to actin filaments on its concave surface, by stabilizing actin filaments \textit{in vitro}.\textsuperscript{47} However, the concave surface also binds to the membrane for the caveolar localization of PACSIN2. Therefore, the role of the actin filament binding of PACSIN2 for caveolar dynamics is an issue that remains to be solved.

\textbf{Phosphorylation of F-BAR domain protein PACSIN2 and caveolar dynamics}

We identified PKC as the kinase that phosphorylates specific sites of PACSIN2.\textsuperscript{20} Surprisingly, the phosphorylation of PACSIN2 by PKC is induced by changes in hypotonic stress and shear stress, which accompany increases in the membrane tension, as well as by chemicals that directly activate PKC. The phosphorylation of PACSIN2 decreased its membrane-binding and tubulation abilities, which could be attributed to the repulsion between the negatively charged, phosphorylated serine 313 by PKC\textsubscript{a} and the relatively abundant negatively charged lipids, such as phosphatidylserine PI(4,5)P\textsubscript{2}. PACSIN2 phosphorylation did not affect its dimerization, auto-inhibition, or dynamin-2 interaction.\textsuperscript{20}

PACSIN2 phosphorylation at serine 313 decreased the lifetime of caveolae,\textsuperscript{20} which resulted in decreases in the number and the stability of caveolae and an increase in the mobility of the caveolae at the plasma membrane. These phenomena could be explained by the removal of PACSIN2 from caveolae, due to its decreased membrane binding.\textsuperscript{20} The removal of PACSIN2 could lead to 2 scenarios, endocytosis and flattening of caveolae, as discussed below (Fig. 2). However, we currently cannot distinguish between these 2 events by TIRFM, because these 2 scenarios are both observed by the disappearance of caveolae.

If caveolae are flattened, then the disappearance of caveolae observed by TIRFM could be explained by the dilution of caveolin-1 from caveolae to the plasma membrane. PACSIN2 regulates the morphology of the necks of caveolae,\textsuperscript{13} and thus the dissociation of PACSIN2 by phosphorylation could make the caveolae deformable into flat membrane.

If caveolae are internalized by endocytosis, then the disappearance of caveolin-1 can be explained by its uptake into the interior of the cell, beyond the illumination of TIRFM. The disappearance of PACSIN2 was followed by the recruitment of dynamin-2,\textsuperscript{20} suggesting that the removal of PACSIN2 provides some spaces for the association of dynamin for membrane scission upon endocytosis.\textsuperscript{20} Independently, the removal of PACSIN2 alone could be predicted to be a trigger for the scission from theoretical approach,\textsuperscript{48} which have shown that BAR proteins have a scaffolding function, involved in stabilizing the neck of the invagination and preventing membrane scission.\textsuperscript{48} Thus, the removal of BAR domain protein from the neck of the invagination could induce membrane destabilization and scission.\textsuperscript{48}

\textbf{Concluding remarks}

Other caveolar proteins, such as cavin1–4, might be co-regulated with PACSIN2 phosphorylation, as cavins bind to and/or recruit PKC\textsubscript{a}.\textsuperscript{49,50} Cavin-1 dissociated from caveolin-1 upon caveola flattening.\textsuperscript{19} Therefore, the mechanical stress-induced removal of PACSIN2 by PKC-mediated phosphorylation would cooperatively function with these caveolar components to promote caveolar endocytosis and flattening. Biomechanical and theoretical analyses of the mechanics of caveolar deformation will facilitate the clarification of the behaviors of caveolae upon various stimuli.

\textbf{DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST}

No potential conflicts of interest were disclosed.

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