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Caveolin-1 activates Rab5 and enhances endocytosis through direct interaction

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ABSTRACT

Caveolin-1, a constitutive protein of the caveolae, is implicated in processes of vesicular transport during caveolae-mediated endocytosis. However, the molecular mechanisms of caveolae-mediated endocytosis are not yet clearly defined. Here, we show the physiological role of the Rab5-caveolin-1 interaction during caveolae-mediated endocytosis. Rab5 was found in caveolae-enriched fractions and Rab5 directly bound to caveolin-1. Furthermore, binding sites of Rab5 to caveolin-1 were identified in the scaffold (SD), transmembrane (TM), and C-terminus (CC) domains, and the Rab5 binding domain of caveolin-1 was required for CTXB uptake. Subsequently, we performed a GST-R5BD pull-down assay to determine whether the Rab5 binding domain of caveolin-1 is involved in Rab5 activity or not. The results showed that overexpression of the Rab5 binding domain of caveolin-1 increase the amount of Rab5-GTP in Cos-1 cells. These findings imply that caveolin-1 controls the Rab5 activity during the caveolae-mediated endocytosis.

Caveolin-1 and caveolin-2 are co-expressed and are most abundant in endothelia, fibroblastic cells and adipocytes, whereas the expression of caveolin-3 is restricted to muscle cells [1,2]. Caveolin-1, a 21–24 kDa integral membrane protein, acts as a scaffolding protein whereby both the N- and C-termini are directed towards the cytoplasm, whilst the central hydrophobic segment inserts into the membrane [3–5]. Additionally it has been reported that caveolin-1 act as a tumor suppressor protein [2,6,7].

Rab5 is a small GTPase that regulates vesicular transportation in the endocytic pathway, where it has been implicated principally in control of vesicle docking and fusion [8]. Emerging data indicate that numerous proteins can selectively bind to Rab5 to facilitate discrete steps in membrane transport [9–13]. Recent studies have postulated two caveolae trafficking routes involving the caveosome and the early endosome. By analyzing the involvement of Rab5 in caveolae trafficking, it was suggested that Rab5 regulates traffic between the early endosome and the caveosome [14].

In previous studies, using a magnetic beads based method combined with immunoprecipitation, we showed that caveolin-1 and Rab5 interacted with TSC2 (tuberin), a tumor suppressor protein, by using anti-tuberin antibody coated magnetic beads [15]. From these results we assumed that Rab5 and caveolin-1 may exist in the same organelle and this interaction could play some role during caveolae-mediated endocytosis. In this study, we determined specific Rab5 binding domains of caveolin-1, and that caveolin-1 binding leads to activation of Rab5. These findings have implications for the Rab5-caveolin-1 interaction during caveolae-mediated endocytosis.

Materials and methods

Cells and materials. Cos-1 cells were kindly provided by Dr. R.S. Yeung (University of Washington, Seattle, WA, USA) cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 1% penicillin, 1% streptomycin (DMEM-10%FBS-1% P.S.). Cos-1 cells were transfected using Lipofectamine 2000 reagent, as described by the manufacturer (Invitrogen). GFP pcDNA3 vector was a kind gift from Dr. Y. Mitsuuchi (Temple University School of Pharmacy, Philadelphia, PA, USA). GST-R5BD vector was kindly provided by Dr. G. Li (University of Oklahoma Health Science Center, Oklahoma City, OK).

Antibodies. Antibodies were obtained from the following sources. Anti-caveolin-1 and anti-rabbit IgG (Santa Cruz), anti-His, anti-GST HRP conjugate (Amersham), monoclonal anti-HA, polyclonal anti-rabbit HA, anti-mouse IgG, and anti-mouse IgG-cy3 (Sigma), anti-Rab5 and anti-EEA1 (BD Bioscience), and GFP (Novus Biologicals).

Expression vector constructs. Full length caveolin-1 cDNA was obtained from total rat lung mRNA using a one step RT-PCR kit

Abbreviation: CC domain, C-terminus domain; SD domain, Scaffolding domain; TM domain, transmembrane domain; CTXB, cholera toxin subunit B.

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(Takara) with specific primers, and cloned into pcDNA3.1. This caveolin-1 cDNA was cloned into pET30a(+) (Novagen) and pcDNA3.1-CFP to construct His-tagged caveolin-1 and CFP-tagged caveolin-1. Each cDNA for the caveolin-1 deletion mutants was amplified by PCR and cloned into a pCMV-HA vector (BD Bioscience).

Wild-type Rab5 (wt) cDNA was amplified by a one step RT-PCR kit (Takara Co. Tokyo) using specific primers, and cloned into pcDNA3.1. Dominant active Rab5 and dominant inactive Rab5 were constructed by mutating wild-type Rab5 cDNA using QuikChange® Site-Directed Mutagenesis Kit (STRATAGEN) with the specific primer, respectively. These mutated cDNAs were then cloned into a pcDNA3-GFP or pcDNA3.1-YFP vector to make either GFP or YFP tagged Rab5. Each Rab5 was also cloned into pGEX-2T vector (Amersham) to obtain bacterially expressing GST-Rab5.

Magnetic beads method. Approximately 1 g of fresh rat spleen was immediately homogenized with homogenization buffer A (20 mM HEPES, 100 mM NaCl, 5 mM $MgCl_2$,1 mM DTT, 0.5 μg/ml leupeptin, 2 μg/ml aprotinin, and 10 μg/ml PMSF, pH 7.5) and centrifuged at 4200g for 20 min and the supernatant was further centrifuged at 105,000g for 60 min at 4 °C. The precipitation was resuspended in phosphate-buffered saline (PBS). This fraction was incubated with magnetic beads coated with anti-caveolin-1 overnight at 4 °C prepared as previously described [15]. Magnetic beads coated with only anti-rabbit IgG were used as a control.

Direct binding assay of caveolin-1 to activated Rab5 analysis. GST, GST-Rab5Q79L, and GST-Rab5S34N were purified from *E. coli.* (BL-21 codon plus (DE3) RIL) according to standard procedures. Purification of recombinant caveolin-1 was based on the Li et al. methods [16]. The purified recombinant caveolin-1 was separated on SDS-PAGE, then transferred to PVDF membrane. His-caveolin-1 was renatured on PVDF membrane by using 8.0–0 M Urea diluted Tris-buffered saline (TBST). Then, the membrane was incubated overnight with TBST containing 5% blocking reagent (accessory for ECL advance product by Amersham Co.) at 4 °C. Subsequently, the membrane was incubated for 1 h with TBST containing 2.0×10^{-2} M active GST-Rab5 (Q79L), inactive GST-Rab5 (S34N) or GST. Furthermore, the membrane was incubated for 1 h with anti-GST HRP conjugate at a dilution of 1:50,000 in TBST and the Rab5-caveolin-1 interaction was detected with ECL advance (Amersham).

Immunoprecipitation. GFP-Rab5 vector and caveolin-1 deletion mutant vector were transfected into Cos-1 cells using the Lipofectamine method (Invitrogen). Thirty-six hours after transfection, Cos-1 cells were washed twice with PBS and solubilized with 1 ml lysis buffer (10 mM Tris, 150 mM NaCl, 5 mM $MgCl_2$, 1% Triton X-100, 0.5 μg/ml leupeptin, 2 μg/ml aprotinin, and 10 μg/ml PMSF pH 7.6). After incubation for 1 h on ice, lysates were centrifuged at 15,000 rpm for 15 min at 4 °C. Two micrograms of monoclonal anti-HA were added to the supernatant and incubated on a rotator for 2 h at 4 °C. Following the incubation, Protein A-Sepharose (SIGMA) was added to the mixture and incubated on a rotator for 1 h at 4 °C. The beads were collected by centrifugation and washed six times with 1 ml of lysis buffer.

Immunostaining. Cells were fixed with 4% formaldehyde in PBS for 10 min. Nonspecific binding of antibodies was blocked by 5% sheep serum for 60 min, after which cells were incubated with primary antibody in 5% sheep serum for 60 min. Bound primary antibodies were visualized with a secondary antibody. After extensive washing with an ECL wash, slide glasses were mounted with IMMU-Mount (Thermo Scientific, Pittsburgh, PA, USA). Cos-1 cells were observed with confocal fluorescence microscopy (OLYMPUS, FV500-IX).

Quantitation of CTXB uptake. Cos-1 cells were plated in 24-well clusters at a density of 4×10^4 cells/cm^2, then transfected 24 h later. Thirty-six hours after transfection, Cos-1 cells were cultured in DMEM–1%P.S. for 30 min at 37 °C. Following incubation, Cos-1 cells were incubated with 0.5 μg cholera toxin subunit B (CTXB) Alexa Fluor 555 for 20 min at 37 °C. The cells were then washed three times in PBS and 500 μl PBS was add to the 24-well plate. Immediately, CTXB uptake was measured with infinite 200 (TECAN).

GST-R5BD pull-down assay. GST-R5BD pull down was based on Liu et al. methods [17]. GST-R5BD was purified from *E. coli* (BL-21 codon plus (DE3) RIL) according to standard procedures. GFP-Rab5 (WT) and Caveolin-1 deletion mutant were expressed in Cos-1 cells after transfection with lipofectamine and incubation at 37 °C for 36 h. Cos-1 cells were washed twice with PBS and lysis for 5 min in 1 ml lysis buffer (25 mM HEPES, pH 7.4, 100 mM NaCl, 5 mM $MgCl_2$, 0.1% NP-40, 2% glycerol, 1 mM DTT, 0.5 μg/ml leupeptin, 2 μg/ml aprotinin, and 10 μg/ml PMSF). Lysis extract was clarified by centrifugation at 10,000g for 5 min at 4 °C, and aliquots (200 μl) of the supernatant were incubated with 20 μl of GST-R5BD bound to the glutathione-Sepharose 4B beads for 10 min at 4 °C under rotation. The beads were subsequently rinsed with lysis buffer, resuspended in SDS sample buffer, boiled for 5 min, and subjected to SDS–PAGE, followed by immunoblot analysis with anti-GFP.

Results

Direct interaction of activated Rab5 to caveolin-1

First, as caveolin-1 is highly expressed in the spleen (data not shown), we thought that this should be an excellent resource for studying the function of caveolae and caveolin-1 binding proteins. By using a combination of immunoprecipitation and a magnetic bead based pull-down method, we enriched a caveolin-1 positive organelle from spleen and observed that Rab5 was present in this fraction (Fig. 1A). These data indicate that Rab5 could interact with endogenous caveolin-1. Furthermore, we conducted a Far-Western blotting analysis using bacterially expressed GST-fused dominant active (Rab5Q79L) and inactive Rab5 (Rab5S34N) with His tagged caveolin-1 (His-caveolin-1) to analyze whether this binding was direct or not. Purified proteins were shown in Fig. 1B. After transferring His-caveolin-1 onto the PVDF membrane, we clearly showed for the first time that activated Rab5 can directly bind to caveolin-1 (Fig. 1C). These data were validated by confocal microscopy analysis as shown in Fig. 1D. When YFP tagged dominant active Rab5 and CFP tagged caveolin-1 were overexpressed in Cos-1 cells, we observed co-localization of Rab5, caveolin-1 and the early endosome marker, EEA-1 (Fig. 1D). In contrast, when dominant inactive Rab5 was transfected, co-localization was clearly diminished (Fig. 1D).

Rab5 binding domain on caveolin-1

We next examined the binding site of Rab5 on caveolin-1. Based on specific domains within the protein we first constructed four different deletion mutants of caveolin-1 fused to HA-tag as shown in Fig. 2A. The amino acids (aa) 1–101 contain the N-terminus cytoplasmic domain with the scaffold domain (SD) domain (cav^{1-101}), whereas aa.1–81 lack the SD domain (cav^{1-81}). To investigate whether the transmembrane (TM) or C-terminal cytoplasmic domain (CC) could bind to Rab5, aa.102–134 ($cav^{102-134}$) and aa.135–178 ($cav^{135-178}$), respectively, were also generated and overexpressed in Cos-1 cells. Expression of all of the caveolin-1 deletion mutants in Cos-1 cells was confirmed by Western analysis using an HA specific antibody (Fig. 2B). After overexpressing mutant caveolin-1, we immunoprecipitated each mutated caveolin-1

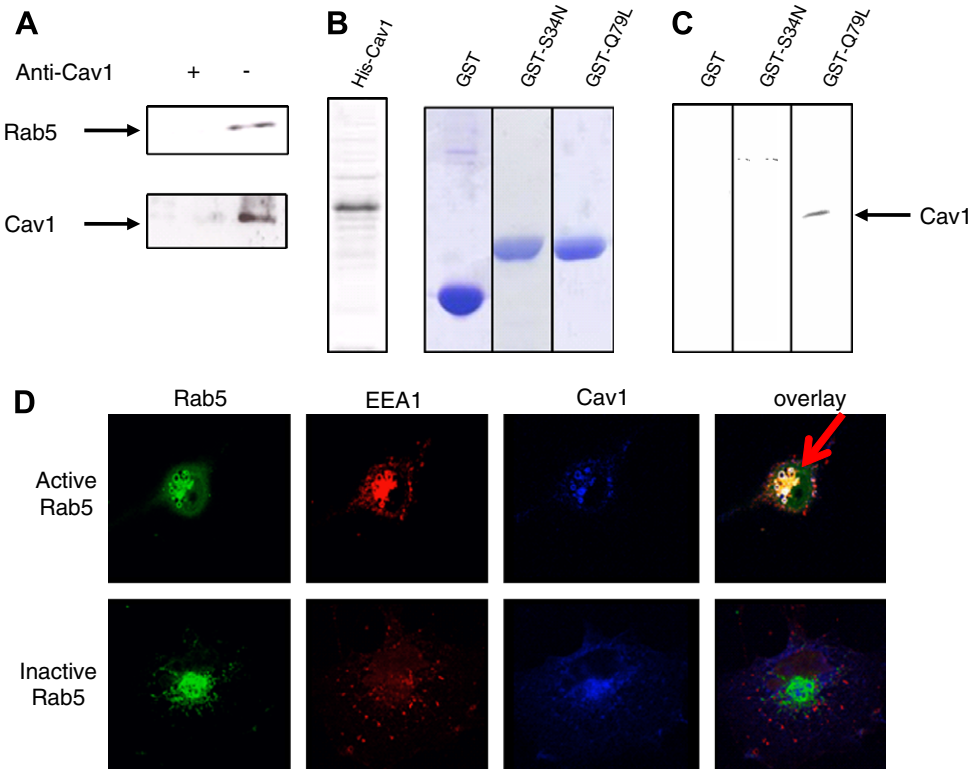

Fig. 1. Identification of Rab5 and direct interaction of Rab5 with caveolin-1. (A) Immunoisolation of caveolin-1 positive microsomes from rat spleen displayed an enrichment for Rab5. Either anti-caveolin-1 IgG-coated Dynabeads (+) or non-coated beads(−) were incubated with rat spleen microsome fraction. Rab5 was detected by Western blotting analysis. (B) Bacterially expressed His-caveolin-1, GST and GST-Rab5 (Q79L and S34N) were purified. (C) After transferring His-caveolin-1 onto the PVDF membrane, GST-Rab5 (Q79L) can directly bind to caveolin-1. (D) CFP-Cav1 was co-expressed with YFP-Rab5 (Q79L) or YFP-Rab5 (S34N), respectively, in Cos-1 cells. Cells were fixed and antibody against EEA1 was used as a specific early endosome marker. We observed co-localization of activated Rab5, caveolin-1 and the early endosome marker, EEA-1 (displayed arrow).

with an anti-HA antibody, then detected for Rab5. As shown in Fig. 2B, the cav$^{1-101}$, cav$^{102-134}$, and cav$^{135-178}$ co-immunoprecipitated with Rab5, whilst cav$^{1-81}$ did not bind to Rab5 (Fig. 2B). Taking into account that cav$^{1-101}$ includes the SD domain compared to cav$^{1-81}$, we predict that the SD domain participates in binding to Rab5. Taken together, this also suggests that amino acid residues 82–178 of caveolin-1 are important for the Rab5-caveolin-1 interaction.

To clarify whether Rab5 binding sites contribute to intracellular localization, we used deletion mutants as described in Fig. 2A, and investigated the intracellular localization of these proteins. We observed that cav$^{1-101}$, cav$^{102-134}$, and cav$^{135-178}$ all co-localized with dominant active Rab5 in Cos-1 cells in Fig. 2C. In contrast, when cav$^{1-81}$ was overexpressed with dominant active Rab5, no co-localization of Rab5 with cav$^{1-81}$ was observed (Fig. 2C). These results correlate well with the fact that the SD, TM and CC domains are Rab5 binding sites on caveolin-1 as shown in Fig. 2B.

Relationship between Rab5 binding domain of caveolin-1 and endocytosis

We then examined correlations between the Rab5 binding domain on caveolin-1 and endocytosis by using CTXB as an endocytosis marker. As shown in Fig. 3A, the uptake of CTXB was higher in cells expressing cav$^{1-101}$, cav$^{102-134}$, and cav$^{135-178}$, compared to control (vector) In contrast, CTXB uptake was not affected in cells expressing cav$^{1-81}$. These results indicate that the SD, TM and CC domains functionally control intracellular traffic of caveolin-1 after they are internalized. Furthermore, we observed that CTXB uptake was influenced by Rab5 activity (Fig. 3B).

Caveolin-1 increases Rab5 activity

Since the Rab5 binding domain on caveolin-1 increases CTXB uptake, we next verified whether these domains affect Rab5 activity by using the Liu et al. methods [17]. We used GST-R5BD (Rab5 binding domain) fusion protein to pull-down Rab5-GTP in cell lysates, followed by immunoblot analysis with a GFP antibody to detect the relative amount of Rab5-GTP. Endogenous Rab5-GTP level was too low to be detected in this assay; thus, we transfected Cos-1 cells with GFP-Rab5 vector to overexpress GFP-Rab5. To investigate the relationship between Rab5-GTP and Rab5 binding domain of caveolin-1, we coexpressed GFP-Rab5 (wild-type) and caveolin-1 deletion mutant, followed by a pull-down assay to determine the amount of Rab5-GTP. Surprisingly, overexpression of Cav1$^{1-101}$, cav$^{102-134}$, and cav$^{135-178}$ increased the Rab5 GTP level (Fig. 4). In contrast, Cav1$^{1-81}$ had no effect on Rab5-GTP level (Fig. 4). These data indicate the Rab5 binding domains of caveolin-1 increase Rab5 activity.

Discussion

In the past, Pelkmans et al. have provided evidence that caveosomes and early endosomes communicate *via* pathway regulated by Rab5 [14]. In this study, we reported for the first time that caveolin-1 directly interacted with Rab5 and determined Rab5 binding domain on caveolin-1. Furthermore, we uncovered caveolin-1 increased Rab5 activity.

Topologically, caveolin-1 is divided into three domains: the N-terminus cytoplasmic domain (residues 1–101) and the C-terminus cytoplasmic domain (residues 135–178) and the hydrophobic

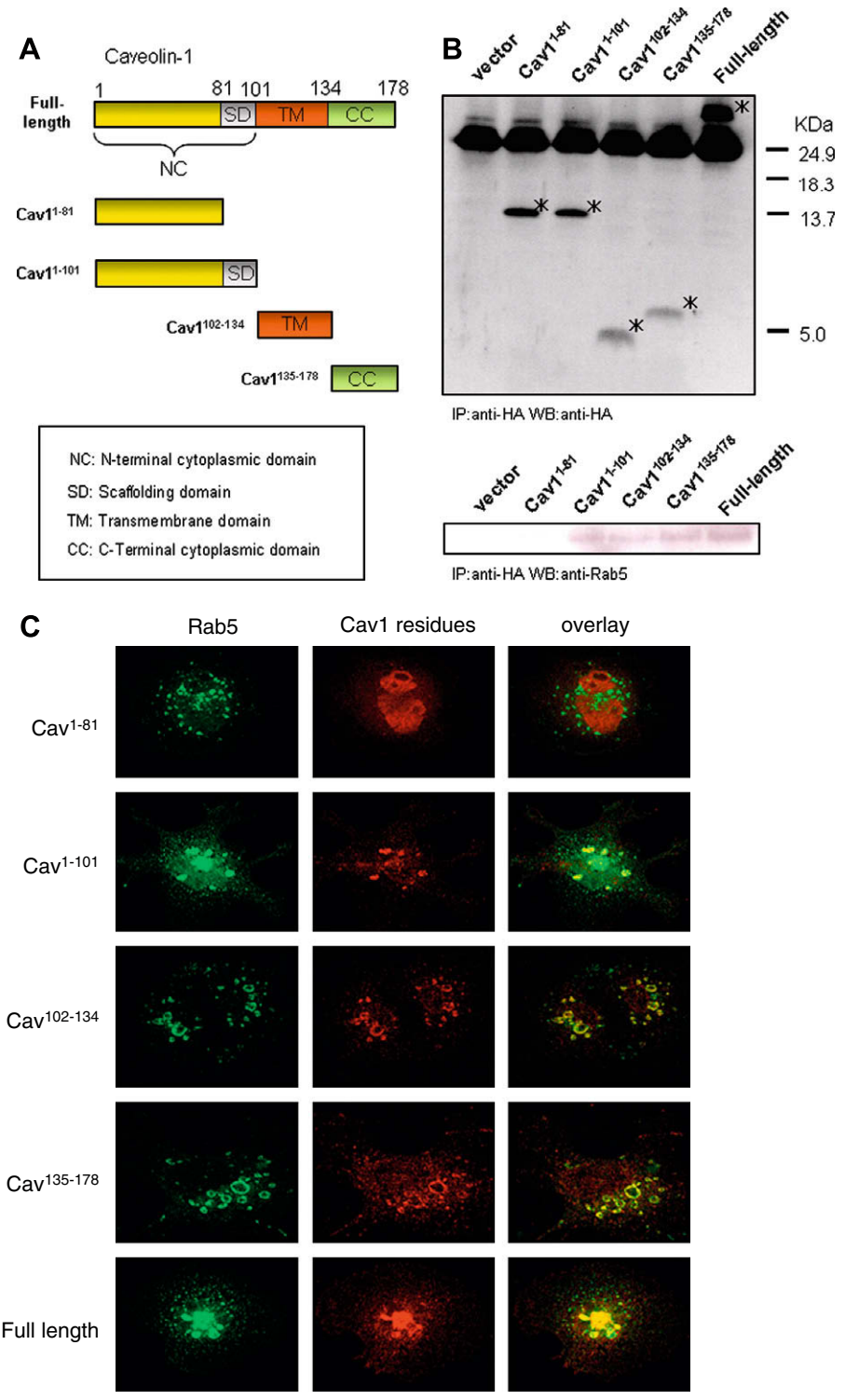

Fig. 2. Determination of the Rab5 binding site on caveolin-1. (A) The N-terminus cytoplasmic domain containing the Scaffolding (SD) domain (residues 1–101: cav$^{1-101}$) and without the SD domain (residues 1–81: cav$^{1-81}$). The C-terminus cytoplasmic (CC) domain (residues 135–178: cav$^{135-178}$) is separated by a hydrophobic transmembrane domain (residues 102–134 cav$^{102-134}$). (B) Each deletion mutant for caveolin-1 and GFP-Rab5 was overexpressed in Cos-1 cells, and each HA-caveolin-1 mutant was immunoprecipitated. Expression were confirmed initially (upper panel, displayed asterisk), and the Rab5 interaction was detected by Western blotting analysis using a Rab5 specific antibody (lower panel). The cav$^{1-101}$, cav$^{102-134}$, and cav$^{135-178}$ co-immunoprecipitated Rab5, whilst cav$^{1-81}$ did not bind to Rab5. (C) Each HA-caveolin-1 deletion mutant was co-expressed with dominant active GFP-Rab5 (Q79L) in Cos-1 cells. Cells were fixed and stained with antibodies against HA and observed under the confocal microscope. The cav$^{1-101}$, cav$^{102-134}$, and cav$^{135-178}$ were colocalized with GFP-Rab5 (Q79L).

transmembrane domain (residues 102–134) [3]. Additionally, the bulk of caveolin-interacting proteins are signaling molecules, and many of them have a common caveolin binding motif that is recognized by scaffold domain (residues 82–101) [2]. Interestingly, we found that Rab5 can interact with caveolin-1 in the region of residues 82–178 (Fig. 2B), which includes the SD domain, TM domain and CC domain. Previous reports have disclosed that the proline-arginine-rich domain (PRD domain) of dynamin-2 could bind to

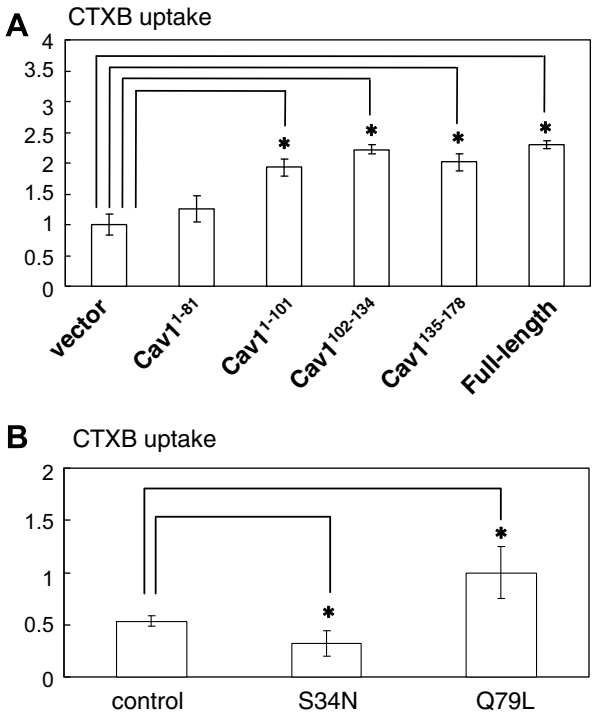

Fig. 3. Functional analysis of distinct caveolin-1 domains and Rab5 by CTXB internalization. (A) Quantification of CTXB uptake in caveolin-1 deletion mutant expressed Cos-1 cells. CTXB were detected as described under "Materials and methods". Error bar indicates SE of three independent experiments (quadruplet for each experiment). $^{*}p < 0.05$. (B) YFP-Rab5 (Q79L), YFP-Rab5 (S34N) or YFP only (control) were overexpressed in Cos-1 cells, and incubated with CTXB for 30 min at 37 °C. CTXB uptake was detected as described under "Experimental procedure". The results show three independent experiments. $^{*}p < 0.05$.

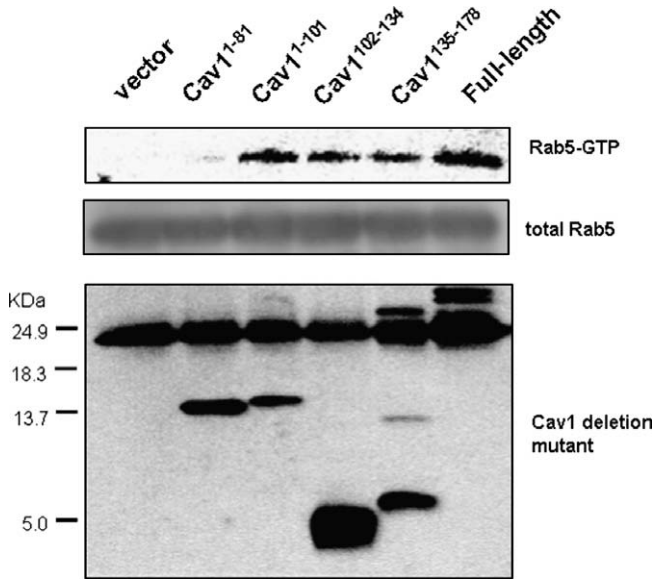

Fig. 4. Rab5 binding domain of caveolin-1 increase Rab5-GTP level. Each HA-caveolin-1 deletion mutant was co-expressed with GFP-Rab5 (WT) in Cos-1 cells. Top, amount of Rab5-GTP in each case as determined by GST-R5BD pull-down assay, followed by immunoblot analysis with the anti-GFP antibody. Middle, total amount of Rab5 in each cell lysate used for the pull-down assay as determined by immunoblot analysis of the lysate directly with the anti-GFP antibody. Bottom, HA-caveolin-1 deletion mutants were indeed expressed in the transfected cells by immunoblot analysis via immunoprecipitation with anti-HA.

amino acid residues 82–178 of caveolin-1 including the SD, TM, CC domains [18]. In addition, protein kinase A (PKA) [19] and connexin 43 [20] could bind to both the SD and the CC domains. Therefore, our date supported TM and CC domain can interact with other proteins.

The activity of Rab5 was measured to understand the biological significance of the Rab5-caveolin-1 interaction. We showed that SD, TM and CC domain are important for activation of Rab5 (Fig. 4). Activation of Rab5 is usually mediated by GDP–GTP exchange factors (GEFs), Rabex-5, Rin1, Rin2, Rin3, Alsin, and ALS2CL, which generate the Rab5-GTP complex [21]. These proteins have been identified that contain a specific, highly conserved domain (Vps9 domain) that catalyzes nucleotide exchange on Rab5. We did not uncover the molecular mechanism of how Rab5 activity is controlled by caveolin-1 in this study. However, one may speculate that caveolin-1 recruits GEF or maintains its activity through direct binding. These hypotheses may be support by the finding in introducing caveolin-1 to 293T cells, which has high efficiency of caveolin-1 expression [22] leads an enlarged endosomes similar the expression of dominant active Rab5 (supplemental Fig. 1). Moreover, these findings may explain how caveolin-1 acts as a tumor suppressor [2,6,7]. For example, previous work showed that change of Rab5 activity by GEF or GAP can direct cells towards tumorigenesis [23,24]. Thus, by controlling Rab5 activity, caveolin-1 may act as a tumor suppressor protein.

In conclusion, we have clearly demonstrated that caveolin-1 directly binds to Rab5 and this interaction is important for the activation of Rab5. Large numbers of molecules have been identified to localize to the caveolae including various receptors and signaling factors and these proteins are endocytosed by caveolae-mediated endocytosis. Further studies will provide new insight into the mechanisms of intracellular signal transduction through caveolae.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2008.10.172.

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