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Adenylyl cyclase localization to the uropod of aggregating *Dictyostelium* cells requires RacC

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Abstract

The localization of adenylyl cyclase A (ACA) to uropod of cells is required for the stream formation during *Dictyostelium* development. RacC is a *Dictyostelium* orthologue of Cdc42. We identified a streaming defect of *racC*[−] cells as they are clearly less polarized and form smaller and fragmented streams. ACA-YFP is mainly associated with intracellular vesicular structures, but not with the plasma membrane in *racC*[−] cells. *racC*[−] cells have a slightly higher number of vesicles than Ax3 cells, suggesting that the defect of ACA trafficking is not simply due to the lack of vesicle formation. While the ACA-YFP vesicles traveled with an average velocity of 9.1 μm/min in Ax3 cells, a slow and diffusional movement without direction with an average velocity of 4 μm/min was maintained in *racC*[−] cells. Images acquired by using total internal reflection fluorescence (TIRF) microscopy and fluorescence recovery after photobleaching (FRAP) analysis revealed that a significantly decreased number of ACA-YFP vesicles appeared near the cell membrane, indicating a defect in ACA-YFP vesicle trafficking. These results suggest an important role of RacC in the rapid and directional movements of ACA vesicles on microtubules to the plasma membrane, especially to the back of polarized cell.

Keywords

RacC; Adenylyl Cyclase; Trafficking; chemotaxis; *Dictyostelium*

INTRODUCTION

Cyclic adenosine monophosphate (cAMP) has important roles in many biological processes such as differentiation, proliferation, and cell motility. In *Dictyostelium*, cAMP has an essential role during the entire developmental program not only as a second messenger but also as a chemoattractant for the directional movement of cells leading to the aggregation [1]. The developmental process of *Dictyostelium* begins with the pulsed secretion of cAMP,

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initiating the multicellular development of *Dictyostelium*. Furthermore, cAMP plays an essential role in the maturation of stalk and spore cells and the germination of spores [2]. In addition, the expression of genes at different developmental stages is also regulated by extracellular cAMP [3]. cAMP is synthesized from ATP by adenylyl cyclase (AC) located on the inner side of the plasma membrane. The expression of the adenylyl cyclase A (ACA) and cAMP receptor 1 (cAR1) is highly elevated by the initiation of the developmental process of *Dictyostelium*. Activation of cAR1 leads to the co-activation of the effectors that control chemotaxis, gene expression for development, and signal relays via the stimulation of expression of ACA and the secretion of cAMP as an extracellular signal for aggregation [2,4,5].

Dictyostelium has three structurally different ACs; ACA, ACB and ACG. ACA is mainly active during aggregation and it is structurally similar to the mammalian ACs [6]. G β γ -subunits of cAR1 activate a PI3 kinase that generates plasma membrane-binding sites for CRAC (cytosolic regulator of AC), which activates ACA [7]. The extracellular concentration of cAMP is strictly controlled through the expression of membrane-bound or extracellular phosphodiesterase and a phosphodiesterase inhibitor that are required for bringing cAMP concentration back to the basal level [8]. The relay of cAMP wave to neighboring cells by the activation of AC is a spatially regulated process. *Dictyostelium* cells directionally migrate to neighboring cells by the chemotactic signal and align in a head-to-tail fashion to form streams. During stream formation, ACA localization shows remarkable enrichment at the back of polarized cells [9]. Localization of the release of cAMP to the uropod of cells is required for the stream formation during *Dictyostelium* development. Although it has been reported that polarized trafficking of ACA vesicles to the rear involves intact actin and microtubule (MT) networks [10], molecular details of this trafficking have not yet been understood.

In this study, we identified RacC as a pivotal regulator of polarized ACA localization at the back of polarized cells during stream formation. ACA-YFP vesicles in *racC*[−] cells maintained slow and diffusional movements without direction and their average velocity was significantly slower compared to wild type cells. Images acquired by using total internal reflection fluorescence microscopy revealed that significantly smaller number of ACA-YFP vesicles appeared near the cell membrane than wild type cells, indicating a defect in ACA-YFP vesicle trafficking in *racC*[−] cells. These results suggest an important role of RacC in the trafficking of ACA vesicles to the plasma membrane, especially to the back of polarized cells.

MATERIALS AND METHODS

Cell Culture and Development

Wild type *Dictyostelium* cells, Ax3, were cultured axenically in HL5 medium supplemented with 60 U of penicillin and 60 μ g of streptomycin per ml. For examining developmental phenotypes, cells were washed twice with 12 mM Na/K phosphate buffer (pH 6.2) and plated in 12 mM Na/K phosphate buffer with 200 μ M CaCl₂ and MgCl₂.

Molecular Biology

The ACA-YFP construct was a generous gift from Dr. Carole Parent (NCI, NIH). ACA-YFP, CFP-RacC, and mRFP-RacC constructs were transformed by electroporation. Transformed cells were selected in the presence of 20 µg/ml G418.

Microscopic Analysis of ACA-YFP localization

Cells were pulsed with 30 nM cAMP every 6 min and then plated on glass-bottomed microwell dishes (MarTek, Inc., Ashland, MA). Images of cells expressing ACA-YFP were acquired at 1.3 sec interval with a spinning disc confocal microscope. We used the Metamorph program (Molecular Devices, CA) to measure the intensity of ACA-YFP, and the number, size, and velocity of ACA-YFP vesicles. Intensity ratios were measured by dividing the intensity value of the membrane with that of the cytosol. All statistics were performed with Prism (GraphPad software, San Diego, CA). A one-way ANOVA using Tukey's multiple comparison test was performed as a post-test to compare the results of *racC* null cell with the results of Ax3 and rescued *racC* null cell. Where indicated, t-tests were also performed.

TIRF microscopy and FRAP analysis

FRAP analysis was performed by using a Zeiss LSM 510 microscope with 514nm Argon laser. A circular region in the membrane was bleached with high-powered bleach pulse and fluorescence was monitored with lower powered scanning beam every 3 seconds. To detect ACA-YFP vesicles reaching the cell periphery, presumably via active trafficking, TIRF images of ACA-YFP in Ax3 or *racC*⁻ cells were acquired with 300 ms exposure at 500 ms interval for 1 min 12 sec using Zeiss Axiovert 200 microscope with a TIRF attachment and 515nm laser.

RESULTS

racC⁻ cells have a polarity defect and do not stream

During aggregation, wild type *Dictyostelium* cells organize into streams that are composed of cells migrating in a head to tail fashion. Localization of the release of cAMP to the uropod of cells is required for the stream formation as *aca*⁻ cells are incapable of generating streams [12]. In addition to defects in chemotaxis as described in a previous study [11], careful examination identified a defective stream formation of *racC*⁻ cells as shown in Figure 1. While Ax3 showed normal streaming at around 8 hours of development, the streaming of *racC*⁻ cells was significantly impaired. *racC*⁻ cells are clearly less polarized and formed small and fragmented streams. This streaming defect was rescued with the expression of CFP-RacC, showing normal streaming at a similar time as Ax3 cells.

Aberrant localization of ACA-YFP in *racC*⁻ cells

ACA has been reported to be asymmetrically localized to the back of polarized cells to attract neighboring cells to the back of cells ahead of them during stream formation [10,12]. To determine if the defect of *racC*⁻ cells in stream formation is due to mislocalization of ACA-YFP, we examined the localization of ACA-YFP in both wild type and *racC*⁻ cells.

ACA-YFP mainly localizes to the uropod membrane and to vesicles in Ax3 cells (Fig. 1B). The most striking difference in *racC*⁻ cells is that ACA-YFP is mainly associated with intracellular vesicular structures, but not with the plasma membrane. Furthermore, sizes of these vesicular structures are bigger than vesicles found in AX3 cells (Fig. 1C). 25% of ACA-YFP-associated vesicles in *racC*⁻ cells have a diameter larger than 0.13 μm^2 , which is hardly observed in Ax3 cells. Measurements of ACA-YFP intensity on the plasma membrane and in the cytosol clearly indicate that trafficking of ACA-YFP to the plasma membrane is significantly impaired in *racC*⁻ cells (Fig. 1E). This defect can be rescued by the expression of wild type RacC or constitutively active form, RacC^{G15V}. The number of ACA vesicles revealed that *racC*⁻ cells have a significantly higher number of vesicles than Ax3 cells (Fig. 1D), suggesting that the defect of ACA trafficking is not simply due to the lack of vesicle formation. These results suggest an important role of RacC in the trafficking of ACA-YFP vesicles to the plasma membrane, especially to the back of polarized cell. To determine if this trafficking defect is specific for ACA, we examined the localization of CAR1-GFP in *racC*⁻ cells. In contrast to ACA-YFP, CAR1-GFP localizes to the plasma membrane with the same level as wild type cells, indicating that the role of RacC in trafficking is specific to ACA. Furthermore, the overall Golgi organization, assessed by Golvesin-GFP [13], was not significantly altered in *racC*⁻ cells (Fig. 1F), suggesting again the role of RacC specifically in the regulation of ACA-YFP trafficking.

Reduced motility of ACA-YFP vesicles in *racC*⁻ cells

Individual vesicles were then traced to determine the path, distance traveled, and velocity of ACA-YFP vesicles. Movements of ACA vesicles were captured for 300 msec at 1 sec interval for 3 min and were traced by using an automatic trace function. Fig.2 shows the paths of ACA-YFP vesicle movements and velocity profiles of individual vesicles in different cell types. As shown, most ACA-YFP vesicles were observed undergoing a diffuse-and-go movement in which diffusional movements are interrupted by directional movements over 0.5 μm . During the rapid and directional movement phase, the speed of the vesicle often reached 0.4–0.7 $\mu\text{m}/\text{sec}$ (Fig. 2A). This is consistent with the speed ($\sim 1 \mu\text{m}/\text{sec}$) of purified molecular motors, such as kinesin or dynein [14]. The ACA-YFP vesicles traveled with an average velocity of 9.1 $\mu\text{m}/\text{min}$ in Ax3 cells. In contrast, ACA-YFP in *racC*⁻ cells maintained slow and diffusional movement without any direction and their average velocity was 4 $\mu\text{m}/\text{min}$. Vesicles in *racC*⁻ cells appear to show a largely random pattern of motion with rare and transient association with MTs, indicated by the absence of movements with high velocity (Fig. 2B). The expression of CFP-RacC or CFP-RacC^{G15V} in *racC*⁻ cells rescued the directional movements. To determine whether the movement and speed observed from these fluorescent vesicles were dependent on intact MTs, we incubated cells with nocodazole (50 μM) for 1.5 h, causing a substantial disassembly of the MT network. This treatment resulted in a severe attenuation in the motility of ACA-YFP vesicles to a velocity similar to *racC*⁻ cells (Fig. 2BC). Attenuation of the motility of ACA-YFP vesicle in both *racC*⁻ and nocodazole-treated cells suggested that reduced motility of ACA vesicles might be due to the lack of vesicle association/transport along with MTs. In contrast, inhibition of actin polymerization by Latrunculin A (5 μM) treatment showed a moderate effect on the vesicle motility, suggesting that ACA-YFP vesicles are mainly transported on MTs.

To examine if reduced motility of ACA-YFP vesicles in *racC*⁻ cells is due to disruption of the MT network, the integrity of the MT network was determined by immunostaining. MT network appeared not to be affected in *racC*⁻ cells (Fig. 3B). If RacC is required for the vesicle association with MT, it is plausible that less ACA-YFP vesicles are associated with MT in *racC*⁻ cells. To determine if ACA vesicles are aligned with MTs in *racC*⁻ cells, we fixed the cells expressing ACA-YFP, stained them with an anti- α -tubulin antibody, and acquired series of z axis images. Images were then improved by performing a computational 3D deconvolution method to check if vesicles were aligned on MTs. As expected, most ACA vesicles align on MTs in Ax3 cells while 30% of vesicles are not proximal to MTs (Fig. 3C). A significant increase of vesicles not proximal to MT (45%) was observed in *racC*⁻ cells, suggesting that RacC might be required for the vesicle attachment to MT. In our previous study [11], we demonstrated that a large fraction of RacC was localized on vesicles. RacC localization on ACA vesicles would be likely if RacC is required for the motility of ACA vesicles. Examination of the subcellular localization of mRFP-RacC revealed partial colocalization of mRFP-RacC on ACA-YFP vesicles. mRFP-RacC was visible on a perinuclear compartment and labeled ACA-YFP vesicles of various sizes (Fig. 3A).

Reduced motility of ACA vesicles presumably results in the slower trafficking of ACA-YFP vesicles to the plasma membrane. We employed a FRAP analysis to examine if the trafficking of ACA-YFP vesicles to the plasma membrane is affected in *racC*⁻ cells. A circular region in the membrane was bleached with high-powered bleach pulse and fluorescence was monitored every 3 seconds. The recovery of ACA-YFP in the bleached area is much slower in *racC*⁻ cells (Figure 4A). The recovery of fluorescence following photobleaching can result from the trafficking of new ACA to the cell surface, recycling of ACA, and/or lateral diffusion of ACA from adjacent stretches of the plasma membrane. A previous study (10) indicated that recovery of ACA-YFP was not largely dependent upon lateral diffusion, suggesting that slower recovery of ACA in *racC*⁻ cells might result from the slower trafficking/recycling of ACA due to reduced motility of ACA vesicles.

Our results indicating the reduced motility of ACA-YFP vesicles were further confirmed by examining the behavior of ACA-YFP vesicles near the cell surface of living cells with a total internal reflection fluorescence (TIRF) microscopy. Total internal reflection fluorescence microscopy is used to detect cellular events near the plasma membrane [15]. The TIRF images were acquired with 300 ms exposure at 500 ms interval for 1 min 12 sec (Fig. 4B–C). ACA-YFP vesicles, upon reaching the cell periphery, moved within a 70-nm plane adjacent to the plasma membrane in a directed manner for distances of microns before fusion. A vesicle fusion event, shown by the fluorescent spot suddenly brightening and vanishing within 300 ms, was occasionally observed (indicated by an arrow in Fig. 4B). However, most vesicles appeared as “newcomer” vesicles that had been absent or only dimly visible before the acquisition. From these images, we counted the number of used or newcomer ACA-YFP vesicles that appeared near the membrane in Ax3 and *racC*⁻ cells. The number of ACA-YFP vesicle appearing near the membrane in *racC*⁻ cells ($\sim 6.4/\mu\text{m}^2/\text{min}$) was much lower than in Ax3 cells ($\sim 11/\mu\text{m}^2/\text{min}$), suggesting significantly reduced trafficking of ACA-YFP vesicles to the membrane in *racC*⁻ cells.

DISCUSSION

Regulation of ACA vesicle trafficking to the membrane, especially at the back of polarized cells to release cAMP to other cells, is a key event for stream formation during *Dictyostelium* development. Our findings show that RacC is required for streaming of cells and for the vesicle trafficking of ACA to the back of polarized *Dictyostelium* cells. During aggregation, the streaming of *racC*⁻ cell was significantly impaired. Asymmetrical localization of ACA-YFP to the back of polarized cells lacks in *racC*⁻ cells due to aberrant trafficking of ACA-YFP vesicles.

We had previously shown that RacC binds to WASP and stimulates its ability to accelerate F-actin polymerization and RacC is preferentially activated at the leading edge of chemotaxing cells [11]. Although the localization of RacC on vesicles is consistent, the role for RacC in the localization of ACA-vesicles in the uropod appears not to be consistent with the previous study. A source of this discrepancy might result from different developmental stages of *Dictyostelium* cells used. Aggregation-competent *Dictyostelium* cells respond to the change of cAMP gradient by protruding a new pseudopod at almost any part of the cell surface. However, cells establish very polarized morphology and ACA localization at the streaming stage and the lateral membrane of cells appear to have very limited ability to initiate a new pseudopod. RacC activity might be required for different processes at different developmental stages.

The role of MTs in vesicle trafficking has been widely demonstrated. MTs serve as tracks for transport of cargo vesicles via the motor proteins dynein and kinesin [16]. Depolymerizing MT with nocodazole caused a cease of movement for secretory vesicles [17]. Similarly, Directional movement of vesicular stomatitis virus G-green fluorescent protein (VSVG-GFP) stopped after nocodazole treatment [18]. This suggests that MTs facilitate the transport of vesicles away from the Golgi. Our finding that RacC is required for the MT-dependent trafficking of ACA is consistent with the previous report demonstrating that the distribution of ACA-YFP on the cell periphery becomes uniform upon nocodazole treatment [10]. *racC*⁻ cells have a slightly higher number of vesicles than Ax3 cells, suggesting that the defect of ACA trafficking is not simply due to the lack of vesicle formation. Although *racC*⁻ cells have almost similar MT structure as AX3 cells, Ax3 cells treated with nocodazole revealed a similar number of ACA vesicles as that in *racC*⁻ cells. This suggests that ACA vesicle trafficking might be related with RacC on MTs. We found that RacC partially colocalized on ACA vesicles. Actin also contributes to vesicle motility in distinct ways by serving as a track for the myosin family of motor proteins [19,20]. The motility of secretory granules in PC-12 cells was found to be mediated by the actin cytoskeleton [21]. Latrunculin A (LatA)-treated *Dictyostelium* cells rapidly round-up and stop moving, and the distribution of ACA on the cell periphery concomitantly becomes uniform, indicating actin-dependent processes are required to maintain the asymmetrical enrichment of ACA [10].

RacC activity appears to be important for the rapid and directional movement of ACA vesicles on MT. Several studies have implicated the role of Cdc42 in vesicle trafficking, including post-Golgi transport and exocytosis [22]. Cdc42 was shown to play a regulatory

role in establishing and maintaining cell polarity via controlling vesicle trafficking [23]. Cdc42 was also shown to play a major role in regulating MT organization and dynamics during cell migration. Previous study suggested that Cdc42 is initially required for both polarized membrane protrusion at the leading edge in moving cell and microtubule organizing center-Golgi apparatus (MTOC-GA) orientation to the direction of cell migration [24]. Activated Cdc42 also regulates post-Golgi trafficking of bone-specific proteins from the Golgi complex to the cell surface in osteoblasts via the GEF faciogenital dysplasia protein (FGD1) [25]. RacC might control vesicular trafficking by affecting the attachment and detachment cycle of the motors, in particular by reducing the attachment of motors to MTs, whereas motor function is unaffected. As we showed in this study, RacC does not appear to alter the speed of moving vesicles as the peak speed of vesicles are comparable to that in wild type cells, but it might affect the frequencies of attachment and detachment to the microtubule tracks. Alternatively, RacC might play a regulatory role in the recruitment of a motor protein to ACA vesicles. Many motors have been reported to interact with cargos via indirect associations, through one or more adaptor or scaffolding proteins [26]. Alternatively, motors might associate with membrane lipids, either directly or indirectly. Cdc42, when bound to a coatamer, inhibits dynein binding to COPI vesicles [27]. Rho has been shown to interact *in vitro* with kinectin, a membrane-anchoring protein for kinesin motor proteins that move vesicles along MTs [28]. Consistent with the role of Cdc42 in trafficking, RacC might be involved in the regulation of ACA trafficking via the specific interaction between motor proteins and cargo vesicles.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- *racC*⁻ cells have a polarity defect and do not stream
- ACA-YFP showed aberrant localization in *racC*⁻ cells
- Motility of ACA-YFP vesicles is significantly reduced in *racC*⁻ cells
- Reduced motility of ACA vesicles results in the slower trafficking of ACA-YFP vesicles to the plasma membrane

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Figure 1.

A. Dictyostelium cells lacking RacC have streaming defects. Cells were plated on non-nutrient agar plates and starved for 8 hours. Low magnification images of Ax3, *racC*⁻, and *racC*⁻ cells expressing CFP-RacC taken as the cells are moving toward the aggregation center by chemotaxis. Wild type cells organize into streams that are composed of cells migrating in a head to tail fashion. **B.** Localization of ACA-YFP in Ax3 and *racC*⁻ cells. High magnification pictures of ACA-YFP in Ax3 and *racC*⁻ cells are shown (bar = 10 μm). Aberrant localization of ACA-YFP in *racC*⁻ cells can be rescued by coexpression of CFP-

RacC or CFP-RacC^{G15V} (constitutively active RacC). C and D. Analysis of size and number of ACA-YFP vesicles. (C) Distribution of the size of vesicular structures associated with ACA-YFP. The number of ACA vesicles over 0.126 μm^2 (area) was significantly more than Ax3. (D) The number of ACA-YFP vesicles, which were normalized by 100 μm^2 of cell area. The vesicle number of *racC*⁻ cells was significantly more than Ax3 cells. Cells treated with nocodazole revealed almost same number of ACA vesicles as in *racC*⁻ cell. Data are means $\pm$ s.e.m. (n=20). E. Quantification of ACA-YFP intensity at plasma membrane and in cytosol. The ratio of ACA-YFP intensity at the membrane to intensity in the cytosol is shown. * Statistically significantly differences between means (unpaired t test, $p < 0.05$). F. Localization of CAR1-GFP and golgesin-GFP in Ax3 and *racC*⁻ cells.

Figure 2.

Tracking of ACA-YFP vesicle movements in Ax3 or *racC*⁻ cells by live cell imaging. Images of cells expressing ACA-YFP were acquired at 1.3 sec intervals for 3 min and tracks of vesicles were traced. (A) Three representative paths taken by vesicles carrying ACA-YFP in different cell types are shown. (B) Histograms of the velocity of ACA-YFP vesicles. The speed was calculated from the distance between two successive positions and speeds of vesicles were sorted into the bins by tallying how many speeds there are in each bin range.

(C) Average velocity of YFP-ACA vesicle movement in *racC*⁻, *racC*⁻ cells expressing RacC and RacC mutants, or Ax3 cells treated with nocodazole are shown.

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Figure 3.

(A) Localization of RacC on ACA-YFP vesicles. Representative image of cells expressing both mRFP-RacC and ACA-YFP is shown. RacC and ACA appear to be colocalized on some vesicles. (B) MT staining shows normal MT networks in *racC*⁻ cells. (C) Deconvoluted fluorescent image showing ACA-YFP vesicles (green) and α-tubulin (red) in fixed Ax3 or *racC*⁻ cells. Graph shows the fractions of vesicles that are not proximal to MT.

Figure 4.

A. FRAP analysis of ACA-YFP. Images were taken right before bleaching (pre), immediately after bleaching (bleach), and at every 6 seconds after bleaching. Images of a representative cell at several time points were shown and circles indicate the bleached area. Recovery of fluorescence intensity in the circle was measured and shown in the graph. B and C. TIRF microscopy was used to follow the dynamics of ACA vesicle trafficking to the membrane (B) Time lapse sequences of ACA-YFP images acquired for 60 sec in Ax3 or *racC*⁻ cells are shown. Multiple ACA-YFP vesicles appeared and some of were fused to the

membrane (arrows). (C) Incidents of ACA-YFP vesicle appearance near membrane for 72 sec were marked as dots. The number of ACA-YFP vesicles appearing near the membrane in Ax3 or *racC*⁻ cells was normalized for 100 $\mu\text{m}^2/\text{min}$ and shown as a graph.