Actin binding domain of filamin distinguishes posterior from anterior actin filaments in migrating *Dictyostelium* cells

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Actin filaments in different parts of a cell interact with specific actin binding proteins (ABPs) and perform different functions in a spatially regulated manner. However, the mechanisms of those spatially-defined interactions have not been fully elucidated. If the structures of actin filaments differ in different parts of a cell, as suggested by previous in vitro structural studies, ABPs may distinguish these structural differences and interact with specific actin filaments in the cell. To test this hypothesis, we followed the translocation of the actin binding domain of filamin (ABD<sub>FLN</sub>) fused with photoswitchable fluorescent protein (mKikGR) in polarized *Dictyostelium* cells. When ABD<sub>FLN</sub>-mKikGR was photoswitched in the middle of a polarized cell, photoswitched ABD<sub>FLN</sub>-mKikGR rapidly translocated to the rear of the cell, even though actin filaments were abundant in the front. The speed of translocation (>3 μm/s) was much faster than that of the retrograde flow of cortical actin filaments. Rapid translocation of ABD<sub>FLN</sub>-mKikGR to the rear occurred normally in cells lacking GAPA, the only protein, other than actin, known to bind ABD<sub>FLN</sub>. We suggest that ABD<sub>FLN</sub> recognizes a certain feature of actin filaments in the rear of the cell and selectively binds to them, contributing to the posterior localization of filamin.

**Key words:** photoswitchable fluorescent protein (mKikGR), structural polymorphism

Actin is a ubiquitous cytoskeletal protein that plays important roles in various cellular activities such as cell migration, cell division and intracellular transport in eukaryotic cells [1–4]. Each of the multiple functions of actin is dependent on interactions with specific actin binding proteins (ABPs). Interaction with the Arp2/3 complex, for example, produces a dendric meshwork of actin filaments in sheet-like pseudopods called lamellipods at the front of migrating cells, and polymerization of actin filaments in this dendric meshwork extends the lamellipods forward. The length of lamellipods is controlled by cofilin, which severs and depolymerizes actin filaments at the back of lamellipods. Additionally, actin filaments form a cortical network underlying the cell membrane, and interact with focal adhesions through linker proteins. Myosin II filaments produce a contractile force at the rear of the cell by pulling the network of actin filaments [5,6]. An advance of the leading lamellipods and contraction...
of the rear cooperatively drives movement of an amoeboid cell. Thus, actin filaments interact with various ABPs and perform different functions in a spatially regulated manner in a cell. It is generally believed that the spatially-defined interactions between actin filaments and ABPs are controlled by local biochemical regulation of each ABP, but there are a number of cases in which such simple biochemical explanations are unknown or insufficient.

Notably, the cortical actin network continuously moves toward the rear of a polarized cell during cell migration, in part driven by contraction of actin and myosin in the rear [7–10]. Recent measurements in the cellular slime mold Dictyostelium discoideum demonstrated that the speed of this rearward cortical flow, or retrograde flow, is similar to that of the forward movement of polarized cells [6], such that the cortical actin meshwork is stationary relative to the substrate during movement. Nonetheless, there is a rapid turnover of cortical actin filaments within seconds, and it is not that the same group of actin filaments remain stationary to the substrate [6].

Dictyostelium filamin, an orthologue of human filamin, is a dimeric ABP with actin cross-linking activity. The meshwork of actin filaments cross-linked by filamin is important for cell migration, chemotaxis and mechanosensing [11–14]. Each filamin polypeptide has an actin binding domain (ABD) consisting of two calponin homology (CH) domains at the N-terminus, a rod domain, and a dimerization domain at the C-terminus [15,16]. ABD <sub>FLN</sub> interacts with actin filaments with high affinity allowing ABD <sub>FLN</sub> to be fused with green fluorescent protein (GFP) to visualize actin filaments in vivo [6,17,18]. In polarized cells, filamin is localized in the posterior region [14,19,20]. Moreover, filamin tends to localize at stretched actin filaments in vivo [13]. It is possible that this property contributes to the control of force transmission and rigidity sensing by filamin [11,12,21]. However, it is not known how filamin distinguishes and interacts with specific actin filaments in a cell.

Each actin protomer in a filament assumes one of the multiple structures depending on its nucleotide state, applied mechanical stress and/or interactions with ABP [22–32]. Binding of cofilin induces a cooperative structural change of actin protomers in filaments that involves supertwisting of the helix. This cooperative structural change enhances the affinity of affected actin protomers for cofilin, resulting in cooperative binding of cofilin [26,28,30]. Conversely, stretching actin filaments inhibits their interaction with cofilin but enhances their interaction with myosin II [5,28,33,34]. Additionally, there is some evidence that certain ABPs, including cortexilin [35,36], fimbrin [24,37], and drebrin [29,38], selectively interact with actin filaments of a specific structure. Along this line, we recently showed that binding of the motor domain of myosin II in the presence of ATP induces a conformational change in actin filaments to reduce the affinity for cofilin, while the supertwisted actin filaments induced by cofilin binding has a lower affinity for the myosin motor domain [39]. Thus, the structure of actin protomers in filaments is potentially an important factor for selective binding of ABPs.

Here, we hypothesized that ABD <sub>FLN</sub> accumulates in the rear of polarized cells by recognizing a certain structural feature of specific actin filaments in the rear of cells. To test this hypothesis, we followed the translocation of ABD <sub>FLN</sub> using a photoswitchable fluorescent protein, monomeric kikume green-red (mKikGR), in polarized Dictyostelium cells [40]. Use of ABD, pioneered by Washington and Knecht [19], eliminates the possible contribution of dimeric filamin molecules by recognizing the orthogonal arrangement of actin filaments [2,15,16,21], and may reduce the contribution of biochemical regulation in actin binding because Dictyostelium ABD <sub>FLN</sub> is not known to be influenced by phosphorylation or by other biochemical regulations. Photo-switching of mKikGR from green to red fluorescence by local irradiation with purple light allows observation of translocation of ABD <sub>FLN</sub> from the irradiated area to other places. We found that the majority of red ABD<sub>FLN</sub>-mKikGR molecules generated in the middle of an elongated cell translocated to the cell rear at a much faster speed than the retrograde flow of cortical actin filaments, even though actin filaments were equally or more abundant in the front of cells. The result suggests that ABD <sub>FLN</sub> recognizes a certain feature of actin filaments in the cell rear, and selectively binds to those filaments.

Materials and Methods

Construction of plasmids

pTX/ABD-fluorescent protein: Coding sequences of ABPs of filamin and a-actinin of Dictyostelium discoideum were subcloned into a modified pTX-GFP vector [41], from which sequences coding 8xHis, GFP and myc had been removed. Then, the coding sequence of GFP (from pTX-GFP) or mKikGR (from CoralHue<sup>®</sup> phmKikGR1-MClinker, Molecular and Biological Laboratories) preceded by a linker (GSGGGGS) was inserted downstream of the ABD sequence of pTX/ABD. pDBsr/mCherry-lifeact: Lifeact [42] fused to the C-terminus of mCherry, was subcloned in pDBsr [5], and expressed under the control of the actin15 promoter. pBIG GFP-GAPA: The coding sequence of Dictyostelium gapA was inserted downstream of GFP in pBIG GFP [43].

Cell culture and expression of ABD-fluorescent protein

Wild-type D. discoideum AX2 cells and GAPA null cells [44] were cultured in HL5 medium supplemented with penicillin and streptomycin [45], and transfected by electroporation with the pTX/ABD-fluorescent protein, pDBsr/mCherry-Lifeact and/or pBIG GFP-GAPA as described previously [46]. Transfectants were selected by 20 µg/mL G418 and/or 6 µg/mL blasticidin S in HL-5 medium at 22°C.

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Live cell imaging and photoswitching of mKikGR under a confocal microscope

Cells expressing ABD-fluorescent proteins and/or mCherry-Lifeact were settled on glass-bottomed dishes (Matsunami, 35 mm dish, hole size: 27 mm, uncoated). To obtain polarized cells, the cells were then starved in 10 mM K’-Na’-phosphate buffer (pH 6.4) until chemotaxis started (~11–12 h at 22°C or ~14–16 h at 11°C). To obtain images of flattened cells, the polarized cells were overlaid with a thin agarose sheet, as described previously [47]. The flattened and polarized cells were observed with a confocal microscope (Zeiss, LSM700) equipped with a 100x objective lens (Zeiss, Plan-Neofluar 100x/1.30 Oil Iris). LED lasers (488 nm: 10 mW, 555 nm: 10 mW) were used for scanning the cells, and images were acquired with ZEN imaging software (Zeiss). The duration for a single frame acquisition was 4–5 s, depending on the size of scanned area. The pinhole size was 1 AU (air unit) (1 AU = 74.20 µm for the 488 nm laser, 79.74 µm for the 555 nm laser). To prevent photobleaching and affecting cell motility, the excitation laser power was set at minimum value (0.5%).

Cells expressing both ABD-GFP and mCherry-Lifeact were irradiated with the 488 nm and 555 nm laser light simultaneously, and those expressing ABD-mKikGR were sequentially irradiated with the 488 nm and 555 nm laser light. The resultant green fluorescence from GFP or mKikGR and red fluorescence from mCherry or mKikGR were separated by a variable secondary dichroic beam splitter (Carl Zeiss) and emission filters, and also a differential interference contrast (DIC) image was simultaneously acquired with each frame. For photoswitching of mKikGR, square ties of the images of Figure 1A were measured along the cell cortex (Fig. 1B). To compare the localization of ABD-mKikGR with that of actin filaments more quantitatively, the fluorescence intensities of the images of Figure 1A were measured along the cell cortex (Fig. 1E: purple line, and Supplementary Fig. S3A), and is consistent with the previous study by Washington and Knecht [19].

Next, the localization of α-actinin ABD (ABD-FLN) fused with GFP at the C terminus (ABD-FLN-GFP) via a Gly-based linker (GSGGGS) was observed in polarized Dictyostelium cells with a confocal fluorescence microscope (Fig. 1A and Supplementary Fig. S2). Actin filaments were visualized by expression of lifeact [42] fused with mCherry at the N terminus (mCherry-lifeact). To compare the localization of ABD-FLN with that of actin filaments more quantitatively, the fluorescence intensities of the images of Figure 1A were measured along the cell cortex (Fig. 1B). Although ABD-FLN-GFP colocalized with actin filaments, it localized more intensely in the rear of the cell (Fig. 1B: at ~0 µm) than in the front (Fig. 1B: at ~20 and ~20 µm). This is indicated by the magenta color of the cell front in the merged image (Fig. 1A), as well as by the ratio of GFP fluorescence intensity to that of mCherry (Fig. 1E: purple line, and Supplementary Fig. S3A), and is consistent with the previous study by Washington and Knecht [19].

Next, the localization of α-actinin ABD (ABD-ACTN) was observed (Fig. 1, C and D and Supplementary Fig. S2) to compare with the localization of ABD-FLN-GFP, because ABD-FLN consists of two CH domains and is homologous to ABD-FLN. A previous report showed that accumulation of GFP-ABD-ACTN in actin-rich structures is weaker than that of GFP-ABD-FLN, and discernable localization in actin-rich structures was observed only when the ABD-FLN is in oligomers such as dimers and tetramers [20]. In this study, monomeric ABD-ACTN-GFP showed almost the same distribution as actin filaments along the cortex (Fig. 1, C and D and orange line in E), although the localization signal along the cortex was not very strong relative to that of cytosol (Supplementary Fig. S5, D and H). GFP alone was only diffusely distributed in the cytoplasm (Fig. 1F). These results suggest that ABD-FLN shows strong preference for rear actin filaments, but with 405 nm light (Supplementary Fig. S1). This coefficient and the fluorescence intensity in the green channel at each pixel were used to estimate fluorescence intensity in the red channel that derived from ABD-mKikGR that was not photoswitched by irradiation. This value was subtracted from the fluorescence intensity in the red channel at that pixel.

Observation of fixed cells

Polarized cells prepared as above were permeabilized and fixed by changing the K’-Na’-phosphate buffer (pH 6.4) to PF buffer (10 mM Pipes-KOH pH 6.8, 3 mM MgCl₂, 1 mM EGTA, 1 mM DTT, 0.05% Triton X-100, 0.1% glutaraldehyde, 1% formaldehyde) and incubated for 5 min at 22°C, followed by incubation in 10 mM Tris-HCl pH 7.0, 3 mM MgCl₂, 1 mM EGTA, and 1 mM DTT for 5 min. Then, they were stained for 1 h in PBS containing 3 nM rhodamine-phalloidin (Invitrogen), rinsed in PBS containing 10 mM DTT, and observed with an LSM 700 microscope.

Results

Localization of ABD-GFP

Localization of ABD-FLN fused with GFP at the C terminus (ABD-FLN-GFP) via a Gly-based linker (GSGGGS) was observed in polarized Dictyostelium cells with a confocal fluorescence microscope (Fig. 1A and Supplementary Fig. S2). Actin filaments were visualized by expression of lifeact [42] fused with mCherry at the N terminus (mCherry-lifeact). To compare the localization of ABD-FLN with that of actin filaments more quantitatively, the fluorescence intensities of the images of Figure 1A were measured along the cell cortex (Fig. 1B). Although ABD-FLN-GFP colocalized with actin filaments, it localized more intensely in the rear of the cell (Fig. 1B: at ~0 µm) than in the front (Fig. 1B: at ~20 and ~20 µm). This is indicated by the magenta color of the cell front in the merged image (Fig. 1A), as well as by the ratio of GFP fluorescence intensity to that of mCherry (Fig. 1E: purple line, and Supplementary Fig. S3A), and is consistent with the previous study by Washington and Knecht [19].

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due to ambient light or by spontaneous conversion. To locally photoswitch ABD-FLN-mKikGR in a polarized cell, the area bound by the yellow square in Figure 2A and B was scanned by 405 nm laser light for ~0.4 s. The red fluorescent ABD-FLN-mKikGR generated near the center of the polarized cell spread in the area slightly posterior to the irradiated area and more intensely at the rear of the cell (Fig. 2, A and B). Fluorescence intensity along the cell cortex was measured to reveal the movement of ABD-FLN during this process (Fig. 2, C and D). In addition, the intensity profile of the observed red fluorescence of ABD-FLN-mKikGR was corrected by subtracting the red fluorescence that was unrelated to irradiation with 405 nm light (see Materials and Methods). Since neighboring cells in the same microscopic field not irradiated with the 405 nm laser light did not show an increase in red fluorescence, the 488 or 555 nm laser light did not contribute to the generation of red fluorescent ABD-FLN-mKikGR (Supplementary Fig. S5). Therefore, this procedure extracted the red fluorescence of ABD-FLN-mKikGR generated by 405 nm laser light irradiation in the boxed area (Fig. 2D).

Accumulation of red fluorescent ABD-FLN-mKikGR in the this is not a general property of ABDs consisting of two CH domains.

**Localization of ABD-FLN-mKikGR**

To investigate the mechanism by which ABD-FLN localizes at the rear of a cell, we next followed the translocation of ABD-FLN real time by observing ABD-FLN-mKikGR in polarized cells with a confocal microscope (Fig. 2, A and B and Supplementary Fig. S4). A pair of green and red fluorescence images was obtained by scanning with 488 nm laser light followed by 555 nm laser light. The green fluorescent ABD-FLN-mKikGR, which is the native state of this fluorescent protein in the absence of photoswitching by irradiation with 405 nm light, showed stronger accumulation in the rear of a cell than ABD-FLN-GFP (Fig. 2A; at ~4.9 s). Additionally, weak red fluorescence was also detected in the rear even without photoswitching (Fig. 2B; at ~4.9 s). This is presumably due to very strong accumulation of ABD-FLN-mKikGR in the cell rear. Red fluorescence might derive from native mKikGR that is weakly excited by 555 nm light and bleed through the red emission filter. Alternatively, a small, background fraction of mKikGR was in the red fluorescent state due to ambient light or by spontaneous conversion.

To locally photoswitch ABD-FLN-mKikGR in a polarized cell, the area bound by the yellow square in Figure 2A and B was scanned by 405 nm laser light for ~0.4 s. The red fluorescent ABD-FLN-mKikGR generated near the center of the polarized cell spread in the area slightly posterior to the irradiated area and more intensely at the rear of the cell (Fig. 2, A and B). Fluorescence intensity along the cell cortex was measured to reveal the movement of ABD-FLN during this process (Fig. 2, C and D). In addition, the intensity profile of the observed red fluorescence of ABD-FLN-mKikGR was corrected by subtracting the red fluorescence that was unrelated to irradiation with 405 nm light (see Materials and Methods). Since neighboring cells in the same microscopic field not irradiated with the 405 nm laser light did not show an increase in red fluorescence, the 488 or 555 nm laser light did not contribute to the generation of red fluorescent ABD-FLN-mKikGR (Supplementary Fig. S5). Therefore, this procedure extracted the red fluorescence of ABD-FLN-mKikGR generated by 405 nm laser light irradiation in the boxed area (Fig. 2D).

Accumulation of red fluorescent ABD-FLN-mKikGR in the
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ABD<sub>FLN</sub>-mKikGR mainly moved by diffusion, and specifically interacted with and was trapped by actin filaments in the rear of the cell.

In addition, there were two minor peaks of red fluorescence along both sides of the cell near the irradiation site (white arrowhead at –13.5 µm and black arrowhead at 10.5 µm from the rear end at 0.9 s after irradiation). The cell cortex around –13.5 µm was close to the irradiated area, but was not directly irradiated. Presumably cortical actin filaments in this area trapped a small fraction of red ABD<sub>FLN</sub>-mKikGR generated close by. These minor peaks in polarized cells moved back to the rear relative to the cells at 0.30±0.19 µm/s (mean±S.D., n=7). These velocities are at the same level as the migration velocity of the cells (0.18±0.11 µm/s: measured at the rear end of four cells), and equivalent to the retrograde flow estimated in a previous study.

The irradiated site was ~12 µm away from the rear end of the cell, implying that the red fluorescent ABD<sub>FLN</sub>-mKikGR moved at a velocity faster than 3.1 µm/s. This velocity is ~9 times faster than the retrograde flow of cortical actin filaments in polarized Dictyostelium cells reported previously (0.34±0.15 µm/s) [6]. Ruchira et al. (2004) demonstrated that a diffusion coefficient (D) of GFP in a polarized Dictyostelium cell was 32±6 µm<sup>2</sup>/s [48]. Diffusion time (T<sub>diff</sub>), calculated by an equation (T<sub>diff</sub> ≈ x<sup>2</sup>/2D) was 2.3 s for 12 µm of diffusion distance x. This value is slightly shorter than the interval between the irradiation and the acquisition of the first image (3.9 s), suggesting that the red fluorescent ABD<sub>FLN</sub>-mKikGR mainly moved by diffusion, and specifically interacted with and was trapped by actin filaments in the rear of the cell.

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Figure 2  Translocation of ABD<sub>FLN</sub>-mKikGR. Time lapse images of a polarized cell expressing ABD<sub>FLN</sub>-mKikGR and migrating toward the right (A, B and C). (A) and (B) show the green and red fluorescence images of mKikGR, respectively, and (C) shows the merged images. The region bound by the yellow square was irradiated by 405 nm laser light for 0.38 s immediately after taking the pre-irradiation image (~4.9 s). Scanning for subsequent images were started at 0.9, 5.5, 10.1, and 14.7 s after starting the 405 nm laser irradiation, which was set as time = 0. Scale bar: 10 µm. (D) Profiles of fluorescence intensity along the cell cortex shown by the white arrows in (C), with the 0 µm set at the rear end. The green and magenta lines show fluorescence intensities of ABD<sub>FLN</sub>-mKikGR in the green and red channels, respectively. The cyan lines show red fluorescence intensities of ABD<sub>FLN</sub>-mKikGR after subtracting the fluorescence intensities in the red channel that were unrelated to stimulated photoswitching. The purple vertical bar in the ~4.9 s graph shows the irradiated region on the cell cortex. The white and black arrowheads show two minor peaks along both cell sides near the irradiation site. Two additional cells analyzed in a similar way are shown in Supplementary Figure S4.
study (0.34±0.15 µm/s) [6]. This coincidence suggests that the red fluorescent ABD\_FLN\_mKikGR that was bound to the lateral cortical actin filaments was transported to the rear by the retrograde flow of cortical actin filaments. Apparently, total amounts of red fluorescence of ABD\_FLN\_mKikGR increased gradually over the time course of 15 s after the irradiation (Fig 2, Supplementary Figs. S4 and S5). This is presumably because fractions of newly generated red ABD\_FLN\_mKikGR was initially in the cytoplasm or bound to the dorsal or ventral cortex outside the thin confocal plane, and these fractions gradually became detectable as it bound to the side cortices or transported to the rear end, which was in the confocal plane.

Localization of ABD\_ACTN\_mKikGR

We next followed the translocation of ABD\_ACTN using the same method as above. Green fluorescent ABD\_ACTN\_mKikGR showed stronger localization along the cortex and in the lamellipodia, and weaker distribution in the cytoplasm, than ABD\_ACTN\_GFP (Fig. 3A and Supplementary Fig. S6). Red fluorescent ABD\_ACTN\_mKikGR was undetectably low before irradiation with 405 nm light (Fig. 3B). This is presumably because the local concentration of ABD\_ACTN\_mKikGR never reached the levels of ABD\_FLN\_mKikGR at the rear of a cell, and consequently, the red fluorescence of ABD\_ACTN\_mKikGR rarely exceeded the background. After irradiation with 405 nm light near the center of the cell, red fluorescent ABD\_ACTN\_mKikGR spread along the length of the cell, with slight enrichment along the cortex (Fig. 3, B and C). The red fluorescence was somewhat stronger in the central region at 1.2 s after irradiation, but was nearly uniform along the cell length at 10.4 s. The localization of green and red fluorescence of ABD\_ACTN\_mKikGRs were eventually similar at ~33.4 s after irradiation (Fig. 3D). This localization is in sharp contrast to that of ABD\_FLN\_mKikGR, which was rapidly and specifically translocated to the rear of the cell.

Localization of actin filaments

In polarized cells expressing ABD\_FLN\_GFP, actin filaments were abundant in the lamellipodia, localized along the entire cortex, and enriched in the front and rear (Fig. 1E). However, because ABD\_FLN\_mKikGR showed much stronger posterior accumulation (Fig. 2A) than ABD\_FLN\_GFP (Fig. 1A: top), it was possible that the expression of ABD\_FLN\_mKikGR drove the accumulation of actin filaments at the rear, and ABD\_FLN\_mKikGR simply co-located with these actin filaments. To rule out this possibility, localization of actin filaments was observed in cells expressing ABDs-mKikGR using two methods. First, mCherry-lifeact was co-expressed with ABD\_FLN\_mKikGR, and was observed simultaneously with the green fluorescence of ABD\_FLN\_mKikGR. As shown in Figure 4A, ABD\_FLN\_mKikGR was exclusively localized in the rear of the cell, while actin filaments were localized not only in the rear, but also in lamellipodia at the front. Second, polarized cells expressing ABD\_FLN\_mKikGR were fixed with glutaraldehyde and formaldehyde, and their actin filaments were stained with rhodamine-phalloidin (Fig. 4B). The staining patterns were largely consistent with live-cell imaging of ABD\_FLN\_mKikGR and mCherry-lifeact, except that cytoplasmic staining with rhodamine-phalloidin was very weak.

Localization of actin filaments in cells expressing ABD\_ACTN\_mKikGR was also observed using the same two methods (Fig. 4, C and D). The localization of actin filaments was similar to that in cells expressing ABD\_FLN\_mKikGR or in cells expressing ABD\_FLN\_GFP or ABD\_ACTN\_GFP. These results suggest that the expression of ABD\_mKikGR does not noticeably influence the localization of actin filaments, and it was clear that ABD\_FLN preferentially localizes at the rear of polarized cells even though actin filaments are abundant, not only in the rear of cells, but also in lamellipodia at the front of cells.

GAPA hardly influences the localization of ABD\_FLN

Although ABD\_FLN is a relatively small domain (240 amino acids) with a high affinity for actin filaments, some other protein that is localized at the rear of polarized cells may also bind to ABD\_FLN and mediate its posterior localization. GAPA, an IQGAP-related protein of D. discoideum, is the only other protein that has been shown or suggested to bind to ABD\_FLN [49]. We thus observed the localization of GFP-GAPA in polarized wild type cells, and that of ABD\_FLN in polarized GAPA-null cells (Fig. 5 and Supplementary Fig. S7). GFP-GAPA localized at the lamellipodia and weakly along the cortex in polarized cells, and posterior enrichment of GFP-GAPA was rarely observed (Fig. 5, A and B). Moreover, ABD\_FLN\_mKikGR localized at the rear of polarized GAPA-null cells. When ABD\_FLN\_mKikGR in the middle of a polarized GAPA-null cell was photoswitched by irradiation with the 405 nm laser, red fluorescent ABD\_FLN\_mKikGR was rapidly and specifically localized at the rear of the cell (Fig. 5, C–F), as occurred in wild type cells (Fig. 2). These results demonstrate that GAPA hardly influences the localization of ABD\_FLN in polarized cells.

Discussion

In this study, we revealed two separate mechanisms with distinct velocities for the translocation of ABD\_FLN from the cytosol to the rear of a polarized cell. Even though actin filaments were abundant in the front of a cell, the majority of red fluorescent ABD\_FLN\_mKikGR newly generated in the middle of an elongated cell moved very rapidly to the rear, but not to the front. The speed of this translocation was ~9 times faster than the retrograde flow of cortical actin filaments. In contrast, the speed of the translocation was similar to that of diffusion of GFP, and therefore, we concluded that this translocation depends on diffusion, rather than on active transport. ABD\_ACTN, which is homologous to ABD\_FLN, showed the same distribution as actin filaments, strongly supporting
the argument against the possibility that some other ABD that competes with ABD$_{FLN}$ for actin binding inhibits ABD$_{FLN}$ from localizing at the front of the cell. It is also evident that the physical size of ABD$_{FLN}$-GFP or ABD$_{FLN}$-mKikGR should not hinder its penetration into the actin meshwork at the front of the cell. Taken together, it is suggested that ABD$_{FLN}$ diffusing in the cytoplasm recognized some feature(s) of actin filaments in the rear of the cell and interacted specifically with these filaments (diffusion and specific capture mechanism, Fig. 6A). It is beyond the scope of this study to elucidate the molecular mechanism of the specific binding between ABD$_{FLN}$ and the posterior actin filaments. However, we and others have proposed that certain ABDs can distinguish actin filaments with different conformations. This hypothesis is based on recent discoveries that actin filaments are inherently polymorphic [32,50], and that external stimuli, including interactions with certain ABPs and mechanical tension, further expands the repertoire of conformational variations [22,23,26,27,30,51–54]. Naturally, actin filaments with different conformations would have different affinities for each ABD. Indeed, stretched actin filaments have a lower affinity for cofilin in vivo and in vitro, and a
in a cell, and this contributes to the specific localization of filamin. However, it has been reported that ABD\textsubscript{FLN} even meshwork more stably than the monomeric ABD\textsubscript{FLN} when actin filaments turnover rapidly, oligomeric ABD\textsubscript{FLN} may be transported to the rear of a cell by the retrograde flow of cortical actin filaments more efficiently than monomeric ABD\textsubscript{FLN}. The ABD\textsubscript{FLN}\textsubscript{-GFP} in this study (Fig. 1) might not be significantly assembled to an oligomer and is thus primarily translocated to the rear of the cells by diffusion and a specific capture mechanism. This is because our linker peptide is not hydrophobic and the posterior localization of ABD\textsubscript{FLN} does not appreciably localize there [20]. GFP tends to form a dimer weakly and it is believed that certain linker sequences between GFP and the fusion partner enhance dimerization [20,56,57]. mKikGR is reportedly a monomeric fluorescent protein while the parent KikGR forms tetramers [40]. Therefore, if mKikGR retains a weak tendency to form oligomers, ABD\textsubscript{FLN}\textsubscript{-mKikGR} in this study (Fig. 2) might be assembled to oligomers by the influence of the fused proteins and/or the linker peptide. Since the oligomerized ABD\textsubscript{FLN} can remain tethered to the cortical actin meshwork more stably than the monomeric ABD\textsubscript{FLN}, even when actin filaments turnover rapidly, oligomeric ABD\textsubscript{FLN} may be transported to the rear of a cell by the retrograde flow of cortical actin filaments more efficiently than monomeric ABD\textsubscript{FLN}. The ABD\textsubscript{FLN}\textsubscript{-GFP} in this study (Fig. 1) might not be significantly assembled to an oligomer and is thus primarily translocated to the rear of the cells by diffusion and a specific capture mechanism. This is because our linker peptide is not hydrophobic and the posterior localization of ABD\textsubscript{FLN}\textsubscript{-GFP} was weaker than that of ABD\textsubscript{FLN}\textsubscript{-mKikGR}. This cortical actin flow mechanism is presumably physiologically relevant, however, since the parent molecules, filamin and α-actinin, are naturally dimers.

It is possible that these two mechanisms contribute to the localization of various other ABPs in vivo. In particular, if actin filaments at different sites in a cell have unique structural features caused by biochemical modulation, nucleotide state, ABP binding, tension, twisting or bending, the diffusion and specific capture mechanism can be applied to ABP localization at various sites in a cell. Since differential local-
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Figure 6 Schematic illustration of the two mechanisms of ABD<sub>ret</sub> translocation from the cytosol in the middle to the rear cortex of a polarized cell. (A) Diffusion and specific capture mechanism. The affinity between actin filaments and ABD<sub>ret</sub> differs depending on the position in the cell, as indicated by the graded intensity of the red color of actin filaments. Since the affinity of the rear actin filaments for ABD<sub>ret</sub> is stronger than other actin filaments, ABD<sub>ret</sub> that is rapidly diffusing in the cytoplasm is specifically captured by the rear actin filaments and accumulates there. (B) Cortical actin flow mechanism. Cytosolic ABD<sub>ret</sub> interacts with cortical actin filaments and is carried to the rear of the cell by retrograde flow (black arrows) of the cortical actin filaments. In both schemes, the front of the cell is to the right.

Conflicts of Interest

All the authors declare that they have no conflict of interest.

Author Contributions

K. S. and T. U. conceived and planned the experiments. K. S., A. N. and T. U. performed the experiments. K. S. analyzed the data. K. S., A. N., H. A. and T. U. contributed reagents and materials. K. S., H. A. and T. U. wrote the manuscript. All the authors reviewed the result and agreed the final manuscript.

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