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Distinct roles of profilin in cell morphological changes: microspikes, membrane ruffles, stress fibers, and cytokinesis

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Abstract Here we report the functional importance of profilin in various actin-mediated morphological changes using H119E mutant profilin I, which is deficient only in actin binding. In the case of actin-protrusive structures from the plasma membrane, H119E-profilin was shown to suppress the formation of Cdc42-induced actin microspikes and Rac-induced membrane ruffles. Conversely, Rho-induced stress fiber formation seemed to occur independently of H119E-profilin introduction. Furthermore, H119E-profilin blocked cleavage furrow ingression and subsequent adhesion to the substratum during cell division, a process in which actin plays indispensable roles.

Key words: Actin; Profilin; Small G protein; Cytokinesis; Cell morphology

1. Introduction

The ubiquitous G-actin binding protein profilin is thought to be a key regulator of actin polymerization in cells (reviewed by Theriot and Mitchison [1]). Profilin was first identified as a G-actin sequestering protein. Now, however, profilin is thought to promote actin assembly from the G-actin pool. Further, profilin is involved in the actin-based motility of *Listeria* [2]. It has also been demonstrated that profilin binds not only to actin, but also to phosphatidylinositol 4,5-bisphosphate (PIP2) and to proteins with proline-rich sequences, which are thought to be important in regulating the actin cytoskeleton. We previously made a H119E mutant profilin I that is defective only in actin binding, retaining its ability to bind all other detectable profilin I binding proteins [3]. This H119E-profilin I was thought to block the signaling cascade leading to actin polymerization through profilin by titrating out endogenous, wild-type profilin I from actin regulatory proteins with poly-proline stretches, as demonstrated in the case of microspike formation facilitated by N-WASP, a profilin binding actin regulatory protein [3,4].

In processes in which cells respond to external stimuli, there is accumulating evidence that Rho-family small G proteins, such as Cdc42, Rac, and Rho, are essential regulators in the rapid actin reorganization leading to the formation of filopodia, lamellipodia, and stress fibers [5]. Recently, many effectors of Rho-family proteins have been identified. However, the necessity of profilin for these small G protein-mediated actin filament reorganizations has remained unclear. In cell cycle progression, actin also plays an important role especially in cytokinesis, a process requiring many proteins, including Rho-family proteins, to work in harmony [6]. In this event, profilin is known to be essential in yeast, ameba, and fly [7–10]. However, in mammalian cells, the contribution of profilin to cell cycle progression remains unclear.

Here, we report the use of actin binding-deficient H119E-profilin I to clarify a variety of roles of profilin in actin reorganization processes.

2. Materials and methods

2.1. Microinjection

cDNA encoding V12-Cdc42, V12-Rac, and V14-Rho were cut out from pEF-Bos expression constructs [11], and the cDNAs were inserted into the *Bam*HI site of pGEX-2T (Pharmacia). Recombinant Cdc42, Rac, and Rho proteins and profilins were then obtained by cleavage of the glutathione *S*-transferase fusion protein as described [3,12]. Injection was performed as described previously [13].

2.2. Retrovirus-mediated gene expression

FLAG-tagged wild-type profilin I, and FLAG-tagged H119E-profilin I were subcloned into a pMX vector. Preparation of retrovirus solutions and infections was performed as described [14]. Then, infected cells were serum-starved overnight before PDGF or LPA stimulation. PDGF stimulation was performed at 13 ng/ml for 10 min at 37°C. LPA stimulation was performed at 100 ng/ml for 10 min at 37°C.

2.3. Flow cytometry and video microscopy

HeLa cells were transfected using lipofectamine reagent (Gibco BRL) and subjected to flow cytometry as described [15]. Cytokinesis was then recorded using phase contrast transmitted light microscopy as described [15] in combination with non-toxic Hoechst 33342 (Molecular Probes) staining at a final concentration of 20 ng/ml for visualizing chromosomes [16].

3. Results

3.1. H119E-profilin I suppresses Cdc42-induced microspike formation and Rac-induced membrane ruffling

We injected recombinantly expressed, dominant-active Cdc42, Rac, and Rho (V12-Cdc42, V12-Rac, and V14-Rho, respectively) with wild-type or H119E-profilin I into Swiss 3T3 cells in order to observe the contribution of profilin to cell morphological changes. Injection of V12-Cdc42 into subconfluent Swiss 3T3 cells induced microspike formation (Fig. 1A), as has been shown previously [13]. Co-injection of H119E-profilin I suppressed this V12-Cdc42-induced microspike formation. In contrast, cells co-injected with wild-type profilin I formed microspikes in a similar manner as cells injected with V12-Cdc42 alone, suggesting the importance of profilin I in Cdc42 function (Fig. 1A,C). We then examined the importance of profilin in Rac-induced membrane ruffling by injecting profilins and V12-Rac into serum-starved Swiss

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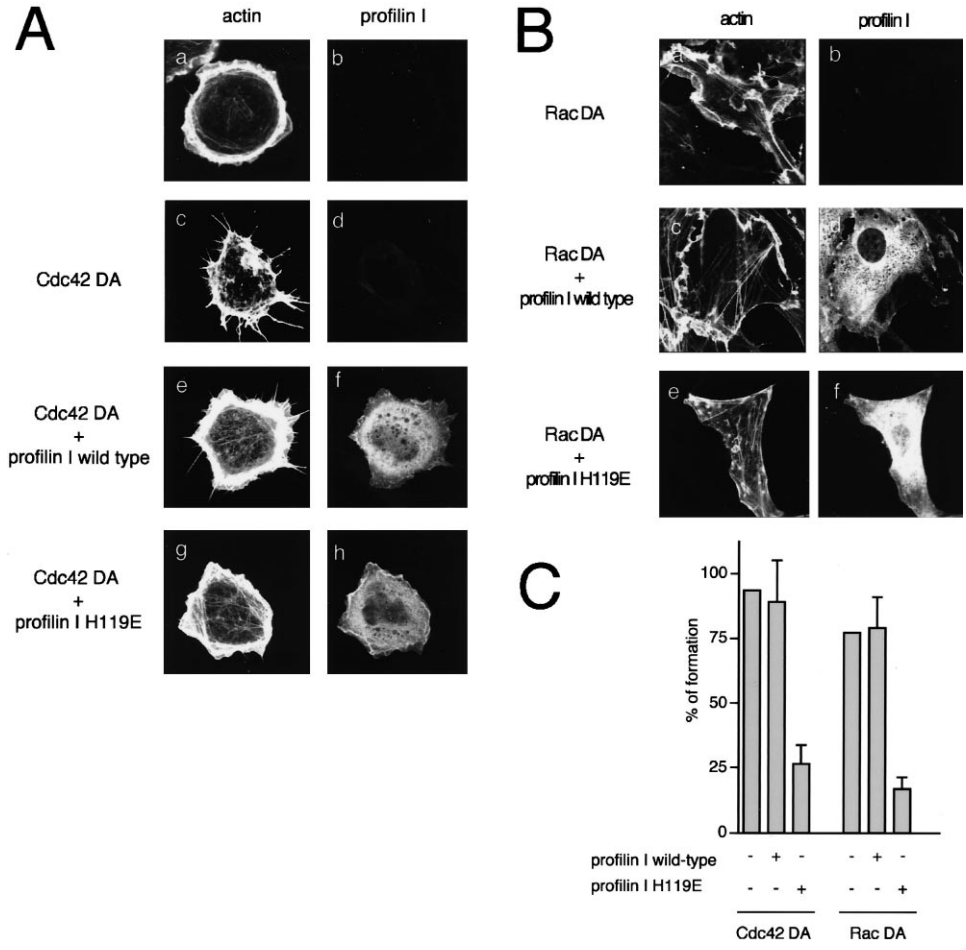

Fig. 1. Actin filament structures of V12-Cdc42 or V12-Rac and wild-type or H119E-profilin I-injected Swiss 3T3 cells. A: Co-injection of V12-Cdc42 and wild-type or H119E-profilin I into subconfluent Swiss 3T3 cells. 4 mg/ml of wild-type or H119E-profilin I were co-injected with 0.5 mg/ml V12-Cdc42 into subconfluent Swiss 3T3 cells. After incubation for 15 min, the cells were fixed and stained. B: Co-injection of V12-Rac and wild-type or H119E-profilin I into serum-starved Swiss 3T3 cells. 4 mg/ml of wild-type or H119E-profilin I were co-injected with 0.5 mg/ml V12-Rac into serum-starved Swiss 3T3 cells. After incubation for 15 min, the cells were fixed and stained. C: The microspike formation rate and membrane ruffling rate in cells injected with Cdc42 or Rac with or without profilins. The cells with microspikes lining the edge were considered microspike formed. The cells with dense actin filament covering more than one third of the edge of injected cell are considered membrane ruffling cells.

3T3 cells. Swiss 3T3 cells co-injected with H119E-profilin I and dominant-active V12-Rac show greatly reduced membrane ruffling compared to cells co-injected with wild-type profilin I and V12-Rac, or cells injected with V12-Rac alone (Fig. 1B,C). Thus, profilin I seems to be important for membrane ruffling induced by Rac.

3.2. *Wild-type profilin I, not H119E-profilin I, suppresses Rho-induced stress fiber formation*

We next examined the effect of profilin in Rho-induced stress fiber formation by injecting profilins and V14-Rho into serum-starved Swiss 3T3 cells that had lost their stress fibers. Surprisingly, cells co-injected with V14-Rho and H119E-profilin I at 4 mg/ml and even at 10 mg/ml showed stress fiber formation similar to that in cells injected with V14-Rho alone (Fig. 2A,B). The cells co-injected with V14-Rho and wild-type profilin I at 4 mg/ml showed thin stress fiber-like actin filament formation at a rate similar to that in cells injected with V14-Rho alone or in cells co-injected with H119E-profilin I and V14-Rho. We then co-injected 10 mg/ml of wild-type profilin I with V14-Rho into serum-starved Swiss 3T3 cells and observed few stress fibers. This high concentration of profilin I suppresses the Rho-induced stress fiber formation (Fig. 2A,B). We conclude from these experiments that profilin plays a limited role in stress fiber formation, and that some mechanism other than profilin will be important for this event.

3.3. *The effect of H119E- and wild-type profilin I on PDGF- and LPA-induced morphological changes*

Next, we prepared Swiss 3T3 cells expressing H119E- or wild-type profilin I using retroviruses and stimulated them for 10 min with platelet-derived growth factor (PDGF) and lysophosphatidic acid (LPA), which are known to cause membrane ruffling and stress fibers in serum-starved cells, respectively (Fig. 3A). In H119E-profilin I-expressing Swiss 3T3 cells, PDGF-induced membrane ruffling was severely impaired compared with that in ectopic wild-type profilin I-expressing cells or control cells, also supporting the importance of profilin in membrane ruffling (Fig. 3A,C). Indeed, the expression of profilin I due to retroviruses was far greater than that of endogenous profilin I (Fig. 3B). Upon LPA stimulation, both

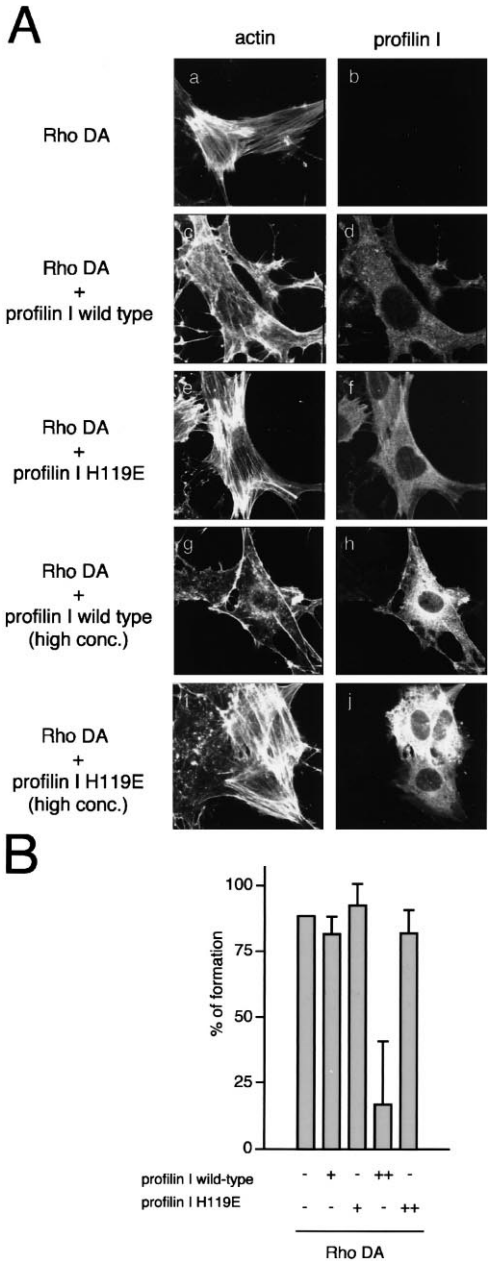

Fig. 2. Stress fibers in V14-Rho and wild-type or H119E-profilin I-injected Swiss 3T3 cells. A: Co-injection of V14-Rho and wild-type or H119E-profilin I into serum-starved Swiss 3T3 cells. 4 mg/ml or 10 mg/ml (indicated as 'high conc.') of wild-type or H119E-profilin I were co-injected with 0.5 mg/ml V14-Rho into serum-starved Swiss 3T3 cells. The cells were fixed after incubation for 20–25 min. B: The formation rates of stress fibers or stress fiber-like actin filaments in cells injected with V14-Rho and various concentrations of profilins. Cells with actin filaments spanning inside the cells uniformly were considered stress fiber bearing.

wild-type profilin I-expressing cells and H119E-profilin I-expressing cells showed similar rates of stress fiber formation, also suggesting a partial role of profilin in stress fiber formation (Fig. 3A,C).

3.4. The effect of H119E-profilin I on cell cycle progression

Profilin has been proved to be essential in cytokinesis in many species except mammals. To identify possible defects in cell cycle progression due to H119E-profilin I expression in mammalian cells, wild-type or H119E-profilin I-transfected HeLa cells were subjected to flow cytometry to analyze the relationship between DNA content and ectopic profilin expression. The results clearly demonstrate a defect in cell cycle progression at the G2/M transition. The population of cells with a 4N DNA content among control cells and those expressing wild-type or H119E-profilin I was 22 ± 1, 33 ± 2, and $46 \pm 2\%$, respectively (means ± S.E.M. from three independent experiments) (Fig. 4A). To investigate at which point in cell division the defect occurs, we performed video microscopy on HeLa cells transfected with wild-type or H119E-profilin I in combination with Hoechst staining to visualize chromosomes (Fig. 4B). The most marked feature in the cell division of H119E-profilin I-transfected cells is that metaphase-aligned chromosomes broaden, partially segregated with nuclear envelope marker lamin B accumulation (not shown) [17], and without any subsequent ingression of the plasma membrane or adhesion to the substratum. This feature was observed among about 50% of the cells recorded with H119E-profilin I transfection. Ectopic wild-type profilin I-expressing cells divide similarly to untransfected cells. The small rise in the G2/M population in wild-type profilin I expressing cells (Fig. 4A) might have resulted from an imbalance between high profilin I expression and actin concentration, because we did not observe any change in wild-type profilin I-expressing cells by immunofluorescence microscopy (not shown) and video microscopy (Fig. 4B). Cells with high profilin I levels are reported to grow or divide normally as observed in this study [18]. Collectively, the above results demonstrate the positive role of profilin in cytokinesis, especially during contractile ring formation and adhesion after division.

4. Discussion

Our results using actin binding-deficient H119E-profilin I strongly suggest that profilin is indispensable for the de novo actin polymerization involved in microspike and membrane ruffling formation. Since H119E-profilin I can bind all detectable profilin I binding proteins except actin, these impairments in actin reorganizations were thought to be the result of the trapping of profilin I binding proteins regulated through Cdc42 and Rac, such as WASP-family proteins and Ena/VASP-family proteins [3,19–21], from endogenous profilin I.

In contrast, profilin seems to play a limited role in LPA- or Rho-induced stress fiber formation. These results were quite surprising, because profilin has been proved to have an actin polymerization promoting effect. However, our observations are consistent with the former observations in some points. First, profilin has actin filament sequestering activity which was the first documented nature of profilin, as observed in the wild-type profilin I injection experiment. Second, in contrast to microspikes and ruffles, it was reported that exogenously added actin was not immediately incorporated into stress fibers. Thus, stress fiber formation might be the result of some changes in already existing filamentous actin structures, not of de novo actin polymerization [22]. Third, both ROCK/Rho kinase and profilin binding protein mDia1 participate in stress fiber formation downstream of Rho. Although the functional importance of profilin for mDia1 in stress fiber formation is not determined yet, it has been suggested that these two effectors of Rho, ROCK and mDia1,

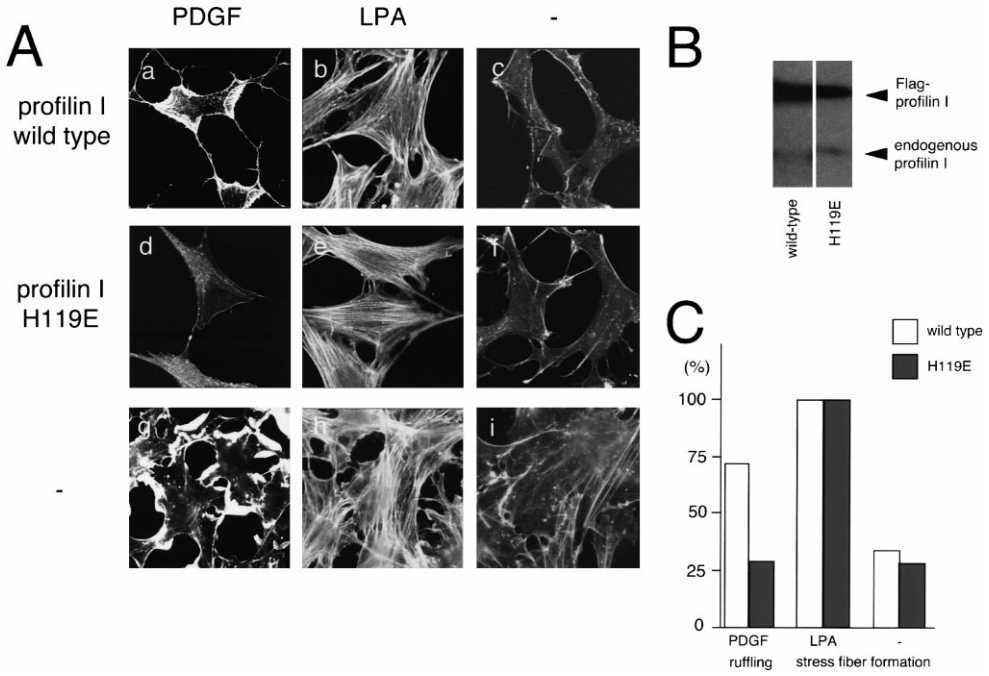

Fig. 3. Effect of profilin on PDGF-induced membrane ruffling and LPA-induced stress fiber formation. A: PDGF (a, d, g) and LPA (b, e, h) stimulation of Swiss 3T3 cells infected with wild-type or H119E-profilin I retrovirus. Swiss 3T3 cells were infected with retrovirus bearing wild-type (a, b, c) or H119E-profilin I (d, e, f). The cells were then analyzed by rhodamine-conjugated phalloidin staining. As a control, uninfected Swiss 3T3 cells were also stimulated (g, h, i). Unstimulated cells are shown in c, f, i. B: Western blotting to examine the amount of retrovirus-mediated expression of profilin I. Lysates of retrovirus-infected cells were analyzed by Western blotting using anti-profilin I antibody. The levels of FLAG-tagged, exogenous profilin I were 5–10 times higher than the level of endogenous profilin I. C: The percentages of membrane ruffling and stress fiber formation due to PDGF or LPA stimulation. At least two independent experiments were performed with similar results as measured in Figs. 1 and 2.

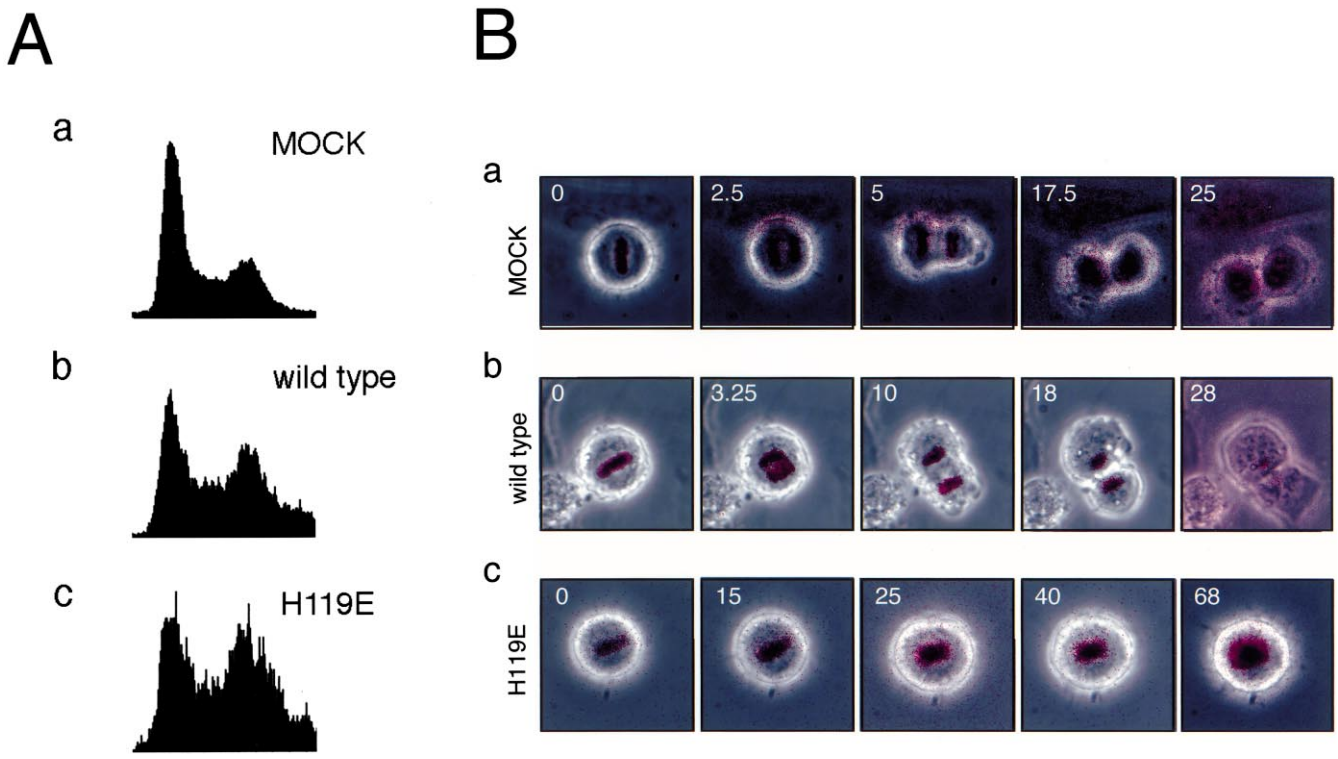

Fig. 4. Effect of profilin on cytokinesis. A: Flow cytometry of the DNA content of mock-transfectant cells (a), and the cells ectopically expressing wild-type (b) or H119E-profilin I (c). B: Video microscopy of mock-transfectants (a) and cells expressing wild-type (b) or H119E-profilin I (c). Times in minutes are shown on the upper right of pictures. Pictures show chromosomes visualized with Hoechst 33342 (red) overlaid with the corresponding phase contrast image.

work in parallel, not in a stepwise fashion, for stress fibers [23]. Thus, it is reasonable that if H119E-profilin I inhibited mDia1 function, stress fibers would be formed through ROCK by regulating myosin-mediated contractility.

In cytokinesis, diaphanous, Cdc12, and Bni1p play a role in cooperation with profilin. Bni1p has been shown to be a binding partner of Rho and Cdc42 [24–27]. In mammalian cells, the roles of the related proteins mDia1 and mDia2 in cytokinesis are unclear [28]. Many kinases such as citron kinase downstream of Rho have also been shown to be essential in cytokinesis [15]. Since H119E-profilin I inhibited cytokinesis, these kinases and profilin might work stepwise downstream of Rho, Cdc42, or other regulatory molecules. Recently, polyproline binding ability of profilin was shown to be important for cell growth in yeast, also supporting the importance of profilin in actin regulatory pathways in cytokinesis [29].

In Fig. 4B, H119E-profilin I appears to inhibit chromosome segregation as well as cytokinesis. The inhibition of cytokinesis due to a defect of profilin usually results in a multinuclear phenotype in yeast and ameba. These organisms do not need to adhere to substratum to proliferate. In cultured cells, profilin was reported to participate in integrin-mediated adhesion to substratum [30]. Further, cooperation between the microtubule system and profilin was reported [31]. Some unknown mechanisms that link between adhesion and microtubules through profilin might control chromosome segregation.

In this study, the use of actin binding-deficient profilin clarified the essential role of profilin in the formation of actin protrusive structures and cytokinesis, but not for stress fibers, suggesting the important role as a transmitter from actin regulatory molecules to actin.

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