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Filamin-regulated F-actin Assembly Is Essential for Morphogenesis and Controls Phototaxis in *Dictyostelium*[*]

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Dictyostelium **strains lacking the F-actin cross-linking protein filamin (ddFLN) have a severe phototaxis defect at the multicellular slug stage. Filamins are rod-shaped homodimers that cross-link the actin cytoskeleton into highly viscous, orthogonal networks. Each monomer chain of filamin is comprised of an F-actin-binding domain and a rod domain. In rescue experiments only intact filamin re-established correct phototaxis in filamin minus mutants, whereas C-terminally truncated filamin proteins that had lost the dimerization domain and molecules lacking internal repeats but retaining the dimerization domain did not rescue the phototaxis defect. Deletion of individual rod repeats also changed their subcellular localization, and mutant filamins in general were less enriched at the cell cortex as compared with the full-length protein and were increasingly present in the cytoplasm. For correct phototaxis ddFLN is only required at the tip of the slug because expression under control of the cell type-specific extracellular-matrix protein A (ecmA) promoter and mixing experiments with wild type cells supported phototactic orientation. Likewise, in chimeric slugs wild type cells were primarily found at the tip of the slug, which acts as an organizer in** ***Dictyostelium*** **morphogenesis.**

Dictyostelium discoideum is a simple model organism for the analysis of multicellular development (1, 2). The cells live as individual amoebae in the soil preying on bacteria. When food is depleted and starvation is imminent, the previously independent amoebae form aggregation streams, which break up into groups of up to 10^5 cells before they form cylindrical migrating slugs. Slugs are sensitive to light, pH, and even slight differences in temperature, which allows them to migrate toward an optimal location for fruiting. Slugs are polar with a tip at the anterior consisting of prestalk cells, whereas the posterior consists predominantly of prespore cells. The tip of the slug senses the light, which controls the migration and phototactic turning (3).

Genetic analysis of slug behavior suggested that as many as 55 genes are involved in phototaxis and that several of the encoded proteins regulate signal transduction pathways involving the intracellular messengers cAMP, cGMP, inositol 1,4,5-trisphosphate, and Ca^{2+} (4–6). This suggests the existence of a fairly complex signaling system. The phototaxis defect in mutants lacking RasD and several RasGEFs points out the involvement of the Ras signaling pathway (7, 8). Escalante *et al.* (9) reported that the BTB protein MigA is necessary for chemotaxis at the single cell level and for slug migration at the multicellular level. Several cytoskeleton-associated proteins such as GRP 125 (10) or villidin (11) and proteins involved in signal transduction and regulation of the cytoskeleton like CAP (12) are also involved in controlling phototactic slug behavior. Furthermore, cells lacking sphingosine-1-phosphate lyase, an enzyme that functions in fatty acid metabolism, showed extremely limited directional migration, and the slugs developed directly at the site of aggregation (13).

Fisher *et al.* (6) and Wallraff and Wallraff (14) tested the behavioral deficits in the slugs of several mutant strains of *D. discoideum* lacking different F-actin-binding proteins. Strains defective in the production of F-actin cross-linking or bundling proteins such as α-actinin, the 34-kDa actin bundling protein, and fimbrin did not show changes in slug behavior. Slugs of mutants lacking filamin (ddFLN), however, migrated shorter distances in the darkness as well as in horizontally directed light. More remarkably, they migrated at an angle of ~45° to the left or right of the incident light, whereas wild type slugs migrated on fairly straight paths toward the light. In these assays therefore ddFLN emerged as the only F-actin cross-linking protein that controls the slug migration.

Filamins are involved in several cellular processes. They function in maintaining the cortical actin network by cross-linking filaments and affect the organization and stabilization of these networks by interwebbing them with membrane proteins and receptors. Furthermore, they are involved in signal transduction and interactions with several components of the NFκB pathway and components of adhesion complexes, and the small GTPases RhoA, Rac1, Cdc42, and RalA have been reported to also interact with filamins (15, 16). Mutations in the human *FLNa* gene are responsible for congenital malformations affecting multiple organ systems, presumably because of defective cell migration during embryonal development (17, 18), and mutations in the *FLNb* gene cause skeletal malforma-

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tions (16, 19). In *Drosophila*, filamin mutations affect oogenesis (20). *Dictyostelium* mutants lacking filamin have defects in the structure of the actin cytoskeleton and exhibit reduced cross-linking of actin filaments, leading, dependent on the parent strain, to reduced size and frequency of pseudopods and resulting in a decreased motility, chemotaxis, and phagocytosis of the mutant cells (21–23).

Structurally filamins are homodimers with large polypeptide chains that associate at their C termini (24, 25). Their conserved N-terminal actin-binding domains (ABD)[2] belong to the ABDs common to the members of the α-actinin/spectrin superfamily of actin-binding proteins (26, 27). The rest of the polypeptide forms six (ddFLN) or 24 (mammalian FLN) repeats of ~96 amino acids, each made up of seven antiparallel β-strands that produce an immunoglobulin fold (28). Their dimerization is mediated via the formation of intermolecular β-sheets between the rod repeats 6 or 24, respectively (29, 30).

The Ig fold domains of the filamins can be mechanically unfolded. Using atomic force microscopy 50–220 pN have been reported; this unfolding is reversible, and the unfolded chains fold back when the external force is removed (31–33). The unfolding of the ddFLN rod was particularly interesting as one of the repeats, repeat 4, unfolded in two steps and refolded along the same pathway (33, 34). Because of the reversible unfolding of their Ig fold domains, filamins can be stretched to several times the length of their native state and could act as extensible linker in the cells and play a mechanical role.

Here we study *Dictyostelium* filamin. Using the filamin-deficient mutant we analyze the efficiency of various mutant proteins to rescue the phototaxis defect and show that only a fully functional molecule can correct the defect.

EXPERIMENTAL PROCEDURES

Dictyostelium Strains and Growth Conditions—*D. discoideum* strain AX2 was used as wild type strain, GHR is a FLN$^-$ mutant generated by gene inactivation through homologous recombination (35), and HG1264 is a FLN$^-$ mutant generated by chemical mutagenesis (21). Wild type and mutant strains were grown at 21 °C in liquid nutrient medium with shaking at 160 rpm (21) or on SM agar plates with *Klebsiella aerogenes* (36). For development in suspension, axenically grown cells were washed twice with Soerensen phosphate buffer, pH 6.0, and resuspended in Soerensen phosphate buffer (17 mM sodium-potassium-phosphate, pH 6.0) at a concentration of 10^7 cells/ml and shaken at 160 rpm at 21 °C. Chimeric mixtures of strains were prepared by harvesting cells grown on *K. aerogenes*, washed free of bacteria, counted, and mixed at desired proportions, and 10^8 cells were plated per phosphate agar plate (17 mM sodium-potassium-phosphate, pH 6.0) or 10^5 cells/water agar plate for phototaxis experiments.

Phototaxis Assay—AX2 and mutant strains were cultivated on *Klebsiella* lawns on SM agar plates. Using sterile toothpicks, the vegetative cells were transferred to 90-mm water agar plates to obtain migrating slugs (6, 37). The plates were wrapped in an opaque black plastic sheet with a slit of ~3 mm in an orientation such that cells were placed furthest away from the slit and incubated at 21 °C. Approximately 48 h after inoculation, slime trails and cellular material were blotted to nitrocellulose filter (BA85, Ø 82 mm; Schleicher and Schuell) by keeping the filter on the plate for 1 h. Thereafter, the filters were stained with 0.1% Amido Black in 25% isopropanol and 10% acidic acid (staining solution) for 10 min followed by incubation in destaining solution (25% isopropanol, 10% acetic acid), twice for 10–15 min to remove the excess stain, washed with water, and air-dried. The filters exhibiting the stained slime trails were used to determine the distance traveled by the slugs toward the source of light from the point of application, and the deviations from the straight line were measured. The angle of deviation was calculated from these two measurements.

Vector Construction—Vectors for expression of full-length ddFLN and its domains as GFP fusion proteins in *D. discoideum* under the control of the actin-15 promoter and actin-8 terminator were constructed using pDEX-GFP (38) and p1ABsr8 (39). The plasmid pDXA-GABD expressing the actin-binding domain as a GFP fusion protein (GFP-ABD) was obtained from Dr. David Knecht (40). To create an ecmA-controlled full-length ddFLN-GFP fusion, a BglII-XhoI insert from FLN-GFP in p1ABsr8 that carries the filamin sequences fused to the GFP sequences but lacks 100 bp from the N-terminal end was cloned in frame into the pDd15-ecmA vector cut with BglII-XhoI (41). The N-terminal 100 bp were retrieved from FLN-GFP p1ABsr8 by BamHI-BglII digestion and cloned into BglII-cut pDd15-ecmA vector already carrying the remainder of the FLN-GFP sequences. CotB::FLN-GFP was constructed by ligating a HindIII-XhoI insert from p1ABsr8 FLN-GFP into the HindIII-XhoI-digested pCotB vector (42). FLNrod2-, FLNrod3-, FLNrod4-, and FLNrod5-GFP corresponded to proteins harboring the ABD and following two, three, four, or five Ig repeats of the rod. FLNΔrod2-GFP lacked repeat 2 (amino acids 346–445), and FLNΔrod4-GFP lacked repeat 4 (amino acids 547–646). The cloning vector was p1AbBsr8 (39). A protein composed of GFP and the complete rod composed of repeats 1–6 (GFP-rod) was expressed from a pDEX-based vector (38). The constructs were generated by PCR and verified by sequencing. In all full-length proteins a linker of four glycine residues separated the GFP moiety from the C-terminal rod repeat to ensure dimerization, which occurs via the last repeat, repeat 6 (25, 29).

The plasmids were introduced into AX2 wild type cells, the ddFLN minus mutants HG1264 or GHR by electroporation. Stable transformants were selected in the presence of 8 μg/ml G418 (Invitrogen) or 3.5 μg/ml Blasticidin S (ICN Biochemicals) as appropriate or both (in case of cotransformation). The transformants were identified by visual inspection under a fluorescence microscope or by colony blotting followed by immunological detection of the expressed proteins in Western blots. In general, the data obtained with transformants of HG1264 are presented.

Fluorescence Microscopy—Axenically grown cells or chimeric slugs developed on phosphate agar plates were fixed in cold methanol at −20 °C. ddFLN was detected using mAb 82–382-8 (21) and actin by mAb act1 (43) followed by incubation with Cy3-labeled goat anti-mouse IgG secondary antibody

[2] The abbreviations used are: ABD, actin-binding domain(s); GFP, green fluorescent protein; mAb, monoclonal antibody; ecmA, extracellular matrix protein A.

(Sigma-Aldrich). Confocal images of live or fixed cells were taken with an inverted Leica TCS-SP laser scanning microscope with a 10× or 63× PL Fluotar 1.32–0.6 oil immersion objective. The 488-nm argon ion laser line was used for excitation of GFP, and the 568-nm krypton ion laser line was used for excitation of the Cy3-fluorophor. The images were processed using the accompanying software. For determination of the distribution of FLN-GFP and GFP-tagged variants of FLN, the cells were kept for 30 min in Soerensen phosphate buffer containing 10 mM EDTA to have cells that were perfectly round. Analysis was done by confocal microscopy. A section through the cells was chosen for analysis, and the intensity was determined using Image J. The highest and the lowest intensities were taken for determination of the ratio. More than 50 cells were analyzed in each case.

Immunoprecipitation of Proteins from Cell Lysates—Axenially growing cells were harvested and washed twice with Soerensen phosphate buffer. Cell pellets resuspended in two volumes of the homogenization buffer (30 mM Tris/HCl, pH 7.4, 2 mM dithiothreitol, 2 mM EDTA, 4 mM EGTA, 5 mM benzamidine, 0.5 mM phenylmethylsulfonyl fluoride, 1 tablet complete mini-protease inhibitor mix (Roche Applied Science)/10 ml, and 30% sucrose) were lysed through Nucleopore membrane filters. The complete lysis of the cells was checked by visual inspection under the light microscope. The lysate was centrifuged at 10,000 × *g* for 25 min at 4 °C. The supernatant was precleared by incubation with protein A-Sepharose beads for 1 h at 4 °C. 600 μl of cleared supernatant were incubated with 100–800 μl of hybridoma supernatant of K3-184-2, an mAb specific for GFP, 325 μl of 5× immunoprecipitation buffer (0.5 M potassium phosphate buffer, pH 7.9, 0.375 M NaCl, 25 mM EDTA, 5 mM benzamidine, 2.5 mM phenylmethylsulfonyl fluoride) and protein A-Sepharose beads at 4 °C for 3 h. The beads were washed three times with 1× immunoprecipitation buffer and then incubated with 5× SDS sample buffer for 5 min at 95 °C. The released proteins were resolved on a 10% SDS-polyacrylamide gel, and the resulting Western blot was probed with mAb 82-421-5 specific for the ABD of ddFLN (21).

Miscellaneous Methods—Western blots were analyzed using the ECL detection system. The native gels were done as described by Schägger (44). Analytical gel filtration analysis was done on a Sephadex G200 column using the SMART system (GE Healthcare). The cells were lysed by freeze thawing, and the 20,000 × *g* supernatant was loaded onto the column. RNA isolation and Northern blot analysis were as described (21). Immunofluorescence methods are described in Ref. 12, and the nuclei were stained with 4,6-diamidino-2-phenylindole. Monoclonal antibodies recognizing ddFLN are described in Brink *et al.* (21), the GFP-specific monoclonal antibody K3-184-2 is described in Ref. 12, and GFP-tagged actin for expression in *D. discoideum* was generated as in Ref. 45.

RESULTS

Properties of Full-length and Truncated Filamin Proteins—In their natural habitat *Dictyostelium* slugs migrate photo- and thermotactically to the soil surface where they form fruiting bodies for spore dispersal in search of a new food depot. Whereas AX2 slugs migrate straight toward the point of light

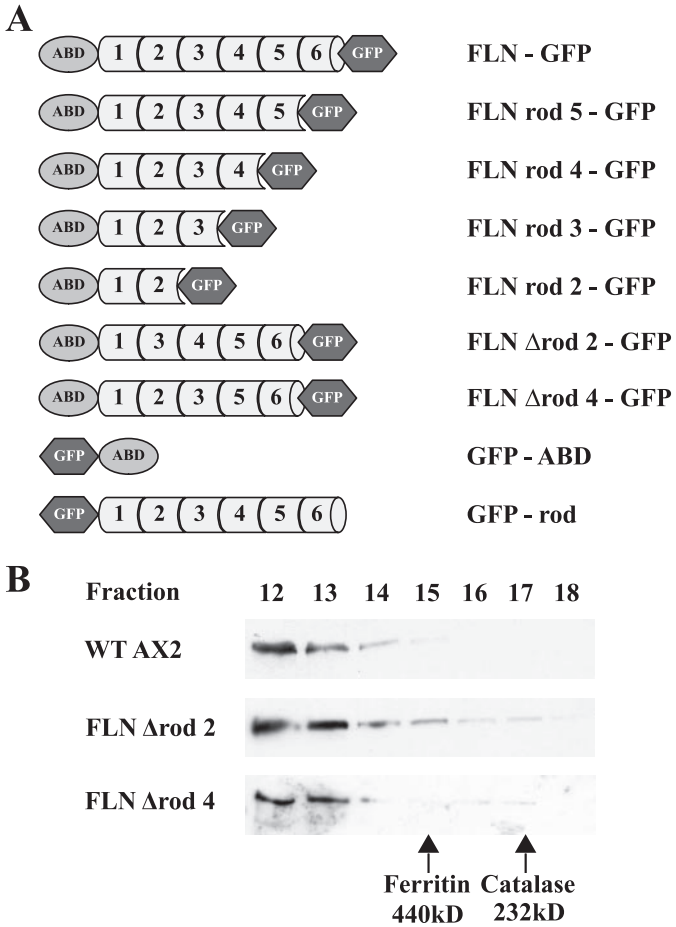

FIGURE 1. **Generation of GFP-tagged FLN proteins.** *A*, schematic representation of GFP-tagged FLN proteins. *B*, C-terminally GFP-tagged FLN proteins behave like native FLN in size exclusion chromatography experiments. The cells were lysed by freeze thawing, and the proteins in the 20,000 × *g* supernatant were separated by size exclusion chromatography (Sephadex G200). The fractions were collected, the proteins were separated by SDS-PAGE (10% acrylamide) and transferred to nitrocellulose, and the resulting blots were probed with mAb 82-454-12 (21) specific for FLN or mAb K3-184-2 (12) recognizing the GFP-tagged FLNΔrod2-GFP and FLNΔrod4-GFP. The proteins eluted from the column in a comparable position and in front of ferritin, indicating their presence in high molecular weight complexes.

entry, mutant slugs lacking filamin were disoriented, and their phototaxis was bimodal (6, 14). To understand the functional role of the protein at the molecular level, we generated plasmids coding for GFP-tagged full-length filamin (FLN-GFP), FLNΔrod2-GFP lacking repeat 2 of the rod domain, FLNΔrod4-GFP lacking repeat 4, the repeat that exhibits an unusual folding behavior, filamins carrying different numbers of repeats (FLNrod2-GFP to FLNrod5-GFP), and GFP-rod, in which GFP replaces the ABD, and expressed them in AX2 and HG1264 or GHR (Fig. 1*A*). We also included pDXA-GABD expressing a GFP-ABD fusion protein in our studies (40).

First we excluded the possibility that the addition of the GFP moiety to the C-terminal residues of the full-length molecule interfered with the dimerization of FLN-GFP. Filamins dimerize through the last repeats that interact with each other to form an extended β-sheet (25, 29). The very last two C-terminal residues in those repeats are preceded by an 8-residue β-strand, both amino acid stretches are far away from the dimerization interface, and the placement of a tag at this end of the protein

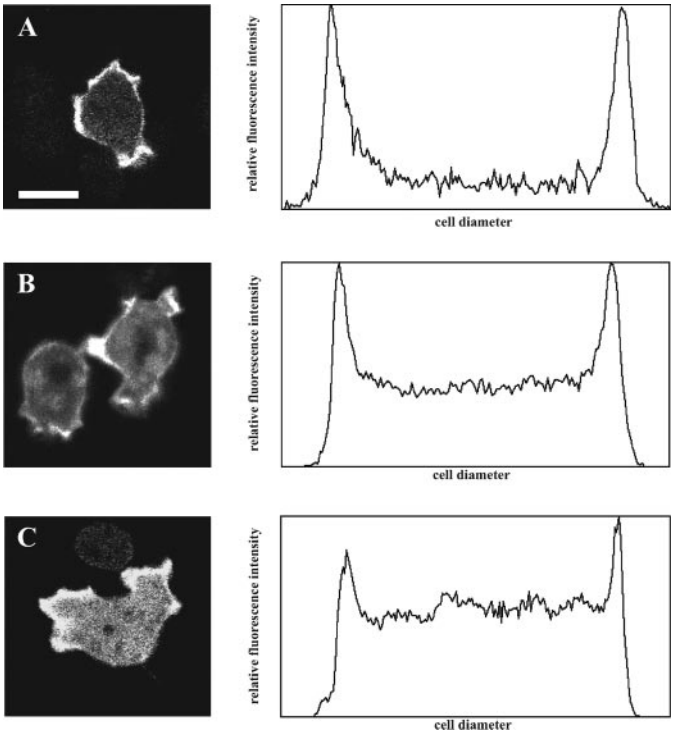

FIGURE 2. **Distribution of GFP-tagged FLN proteins in living HG1264 cells.** *A*, FLN-GFP is primarily present in the cell cortex and less so throughout the cytosol. FLNΔrod4-GFP (*B*) and FLNrod2-GFP (*C*) are found in the cortical region and in cell protrusions and have higher levels in the cytoplasm than FLN-GFP. Life images were acquired using a Leica TCS SP confocal microscope. *Bar*, 10 μm. The distribution of the GFP fusion proteins across individual cells is shown next to the life cell images. For these measurements cells were allowed to round up by incubation in Soerensen phosphate buffer containing 10 mM EDTA. At least 50 cells were analyzed per strain. In HG1264 expressing FLN-GFP the GFP fusion protein was strongly enriched in the cortical region with very low levels in the cytosol, whereas in cells expressing FLNrod-GFP proteins the fusion protein content in the cell interior increased. A typical pattern is shown in each case.

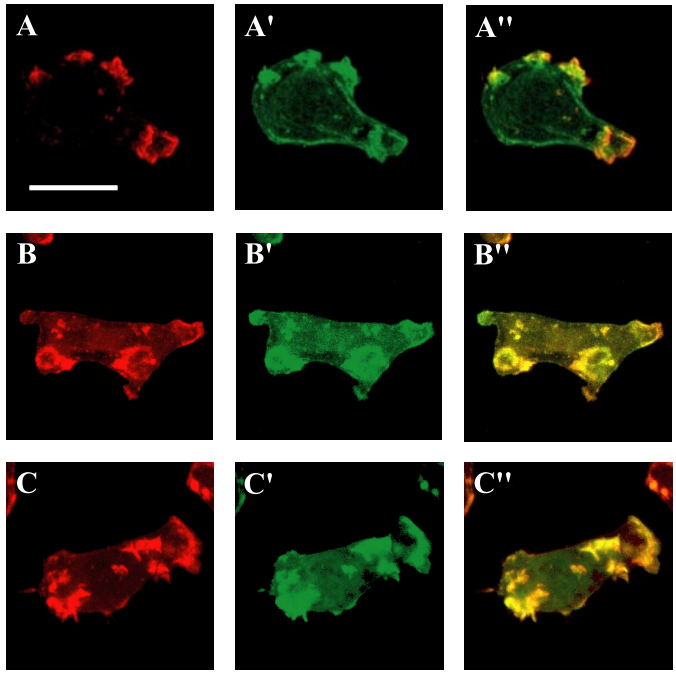

FIGURE 3. **Colocalization of cortical filamin proteins with actin.** In HG1264 transformants FLN-GFP (*A'*), FLNΔrod4-GFP (*B'*), and FLNrod2-GFP (*C'*) colocalized with actin (*A*–*C*). The overlay is shown in *A''*–*C''*. The cells were fixed with cold methanol and stained for actin using mAb act1. *Bar*, 10 μm.

should not interfere with dimerization. To minimize any interference even further, we separated the GFP tag from the filamin sequences by a short linker peptide comprised of four glycine residues. In native gel electrophoresis filamin and GFP-tagged filamin migrated at a position larger than 200 kDa (data not shown), and in size exclusion chromatography, where we separated proteins from whole cell lysates, native filamin (FLN), FLNΔrod2-GFP, and FLNΔrod4-GFP eluted in a comparable fashion and as high molecular weight complexes from the column (Fig. 1*B*). We therefore assume that the GFP-tagged proteins do not have disturbed dimerization behavior.

Analysis of living and fixed cells showed that all GFP-tagged fusion proteins with the exception of GFP-rod were present in the cytosol and at the cell cortex and accumulated at leading fronts during cell movement. We also noted that full-length FLN was more prominent at the cell cortex and less enriched in the cytosol as compared with the modified FLN proteins (Fig. 2*A*). This was confirmed in a quantitative analysis where we measured the relative fluorescence intensity across the cells. FLN-GFP fluorescence at the cell periphery was six times stronger than inside the cell, for FLNΔrod4-GFP we noted a 1.9-fold enrichment at the periphery, and for FLNrod2-GFP we noted a 1.7-fold peripheral enrichment (Fig. 2, *B* and *C*). GFP when expressed in strain AX2 is diffusely distributed throughout the cells and does not show an enrichment in particular places as reported before (45). In fixed cells the cortical staining coincided largely with the actin staining (Fig. 3). For GFP-tagged rod, which lacks the ABD, we observed a difference in localization in wild type and mutant cells. In AX2 cells its distribution paralleled the one observed for FLN-GFP, whereas in FLN$^-$ cells it was present in the cytoplasm in a homogeneous fashion and was not enriched at the cortex (Fig. 4, *A* and *B*). The different distribution in wild type *versus* mutant cells was explained by the formation of heterodimers with the endogenous protein in AX2 that targeted the fusion protein to the cell cortex. In coimmunoprecipitation experiments we could precipitate endogenous filamin together with GFP-rod using the GFP-specific mAb K3-184-2. Probing the immunoprecipitate with mAb 82-421-5 specific for filamin ABD, we detected a protein of 120 kDa corresponding to filamin (Fig. 4*C*).

We conclude from these observations that the ABD targets DdFLN to the cell cortex and the cortical actin cytoskeleton; however, there appear to be additional determinants in the molecule that contribute to the correct localization as taken from the results obtained with truncated filamins (Fig. 2, *B* and *C*).

F-actin Cross-linking by Filamin Is Essential for Dictyostelium Phototaxis—Dictyostelium cells are able to sense light both as single cells as well as in the multicellular slug stage, and wild type slugs migrate straight toward a light source (Fig. 5*A*). Filamin-deficient strains have a strong phototactic defect and migrate in an angle toward the light source and have a shorter migration path (for comparison see Fig. 7*C* and Fig. 1 in Ref. 6; ABP120-deficient). In phototaxis assays, HG1264 slugs expressing FLN-GFP showed a behavior like AX2 wild type and

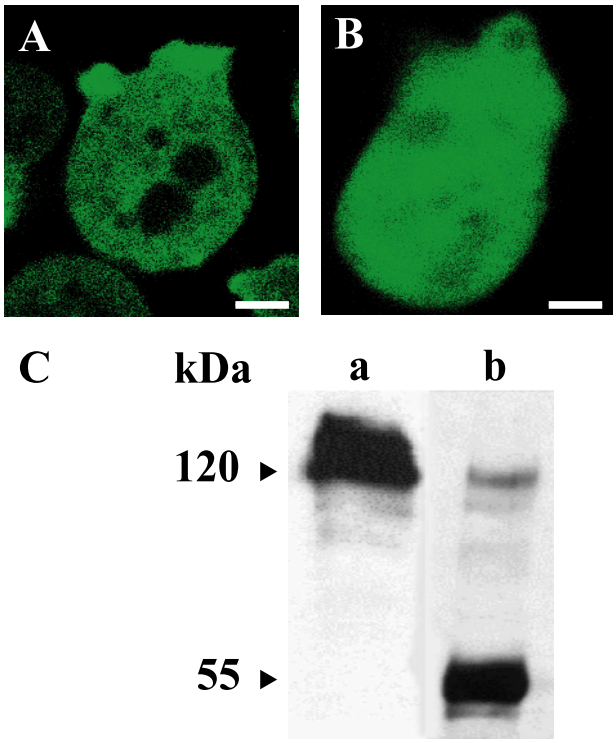

FIGURE 4. **GFP-rod shows a different distribution in AX2 *versus* HG1264.** *A*, in AX2 cells GFP-rod is observed throughout the cell and accumulates in cell extensions in a similar manner as the full-length protein. *B*, in HG1264 GFP-rod is evenly distributed throughout the cells and does not accumulate in cell fronts. *Bar*, 5 μm. *C*, GFP-rod dimerizes with endogenous FLN in AX2 cells. GFP-rod was immunoprecipitated from AX2 transformants with GFP-specific mAb K3-184-2. *Lane a*, FLN is detected in the whole cell homogenate at 120 kDa (*arrow*) with mAb 82-421-5. *Lane b*, the immunoprecipitate was probed with the ABD-specific mAb 82-421-5 to detect endogenous FLN. The signal at 55 kDa corresponds to the heavy chain of the antibody. The proteins were separated in SDS-PA gels (10% acrylamide).

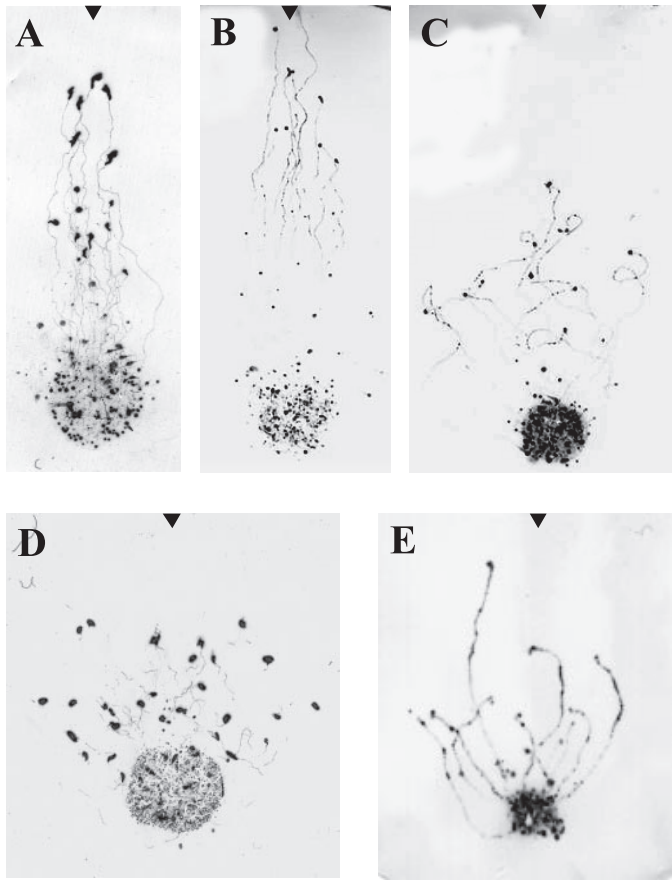

FIGURE 5. **F-actin cross-linking by filamin is necessary for normal phototaxis.** Expression of FLN-GFP in HG1264 (*B*) restores phototaxis completely and slugs migrate straight toward the light like AX2 wild type slugs (*A*). *C*, HG1264 slugs expressing FLNrod5-GFP have a slightly improved migration behavior and have longer migration trails; however, they migrate in a wide angle toward the light. Expression of FLNΔrod4-GFP (*E*) and FLNΔrod2-GFP (*D*), molecules that can cross-link actin filaments but are missing internal repeat 2 or 4, do not rescue the phototaxis defect either.

migrated in a straight path toward the light (Fig. 5*B*), whereas mutant slugs expressing FLN proteins carrying a shortened rod (FLNrod5-GFP to FLNrod2-GFP) migrated like the parent strain HG1264 in a wide angle toward the light showing no improvement in phototactic orientation, although the migration paths were longer (Fig. 5*C*). Molecules lacking internal repeats, namely FLNΔrod2-GFP and FLNΔrod4-GFP, did not rescue the phototactic defect either (Fig. 5, *D* and *E*). GFP-rod and GFP-ABD on their own or when coexpressed in the mutant were also not able to rescue phototactic migration (data not shown). These results suggest that the presence of filamin molecules in the actin cortex is not sufficient for functioning in phototaxis. Rather, only the full-length protein, which can cross-link actin filaments into correct three-dimensional assemblies, can rescue phototactic orientation.

Expression of ddFLN in Prestalk Cells Rescues the Phototaxis Defect in the Mutant—Slugs consist mainly of two cell types: prespore and prestalk cells. We therefore tested in which cell type filamin function is needed for normal phototaxis. To address this we expressed filamin under the control of the ecmA promoter, a prestalk cell-specific promoter, and the prespore cell-specific cotB promoter. The ecmA promoter is strictly expressed at the anterior one-tenth portion of the slug (Fig. 6*A*), and expression of the cotB promoter is restricted to the posterior two-thirds of the slug (41, 42). After culmination, the cells in the rear of the slug eventually will form spores. Upon cell type-specific expression we found that only expression of FLN-GFP under the control of the ecmA promoter rescued the phototactic defect completely, whereas slugs expressing filamin-GFP under the cotB promoter showed the mutant phenotype in phototaxis (Fig. 7, *A* and *C*).

Filamin Function Is Necessary for Tip Formation—Filamin-deficient mutants undergo a normal development and follow the morphogenetic program. They have a tip as the morphogenetic organizer and form migrating slugs. However, the tip does not correctly function in phototactic orientation. The phototaxis rescue by ecmA::FLN-GFP raises the possibility that the expression of ddFLN in pstA cells may help in sorting out this cell type to the tip during *Dictyostelium* morphogenesis. To test this, we mixed wild type and mutant cells in various ratios. The use of actin-GFP expressing wild type cells allowed us to distinguish both strains easily. We observed that 30% of AX2 cells in a chimeric mixture with HG1264 rescued the phototaxis defect, suggesting that ddFLN is necessary for cell sorting (Fig. 7*B*). Microscopic images of the chimeric slugs showed that the AX2 cells accumulated in the anterior tip in a location similar to the one of ecmA::FLN-GFP-expressing cells (Fig. 6*B*). In control

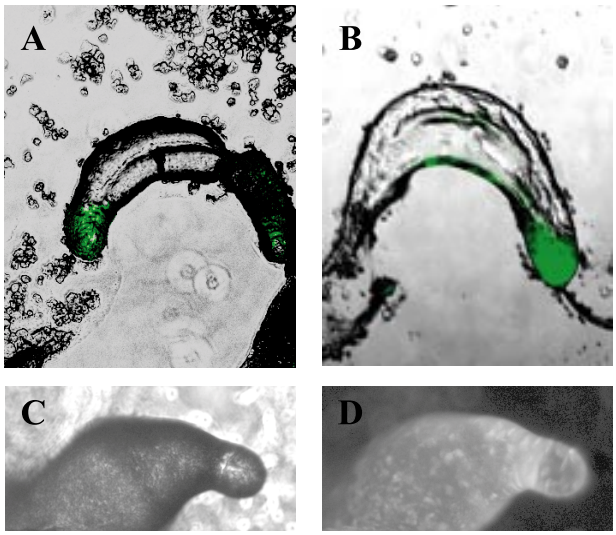

FIGURE 6. **Expression of FLN at the tip of the slug.** *A*, expression of FLN-GFP in HG1264 under control of the ecmA promoter shows the presence of the FLN-positive cells at the tip. *B*, in mixing experiments AX2 wild type cells sort out from HG1264 cells and assemble at the tip. For the sorting experiment we mixed 10% AX2 cells expressing a GFP-tagged actin with 90% HG1264 cells. *C* and *D*, to exclude an effect of the actin-GFP on the sorting process, we mixed AX2 wild type with AX2 cells expressing GFP-actin (70 to 30 ratio). The GFP-tagged cells were present throughout the slug. *C*, phase contrast. *D*, fluorescence image. In *A* and *B*, phase contrast and fluorescent image are overlaid.

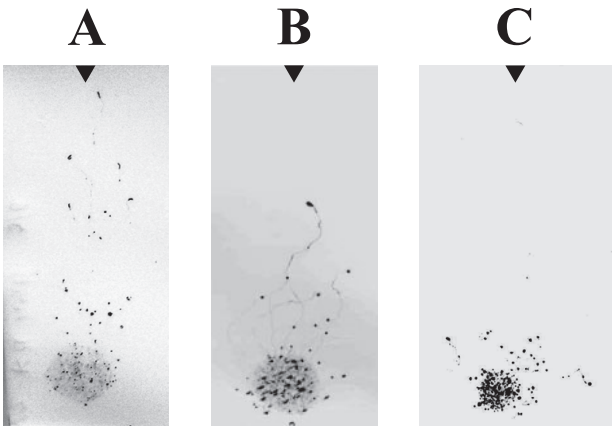

FIGURE 7. **Expression of FLN is only required at the tip of the slug for correct phototaxis.** *A*, expression of FLN-GFP in HG1264 under control of the ecmA promoter rescues the phototactic defect and slugs migrate straight toward the light source. *B*, in mixing experiments AX2 cells sort out from HG1264 mutant cells and assemble at the tip (Fig. 6*B*) restoring phototaxis of the slug. The ratio of wild type to mutant cells was 30:70. *C*, expression of FLN-GFP in HG1264 under control of the cotB promoter, which is active in the posterior region of the slug, does not rescue the phototaxis defect.

experiments we excluded that the expression of actin-GFP affected the localization of these cells in slugs. When we mixed AX2 with AX2 expressing GFP-actin, we found that the GFP-tagged cells were distributed throughout the slug and were not specifically enriched in the posterior or anterior part of the slug (Fig. 6, *C* and *D*).

DISCUSSION

Filamin-deficient cells can undergo normal development and form normal looking fruiting bodies consisting of a stalk and a spore head, indicating that the processes involving differentiation into prestalk and prespore cells and that their correct sorting along the anterior-posterior axis takes place (21). This implies that as multicellular development proceeds, a tip is formed that acts as signaling center and produces cAMP waves passing through the mound. The tip functions in the multicellular stages of *Dictyostelium* in a way similar to morphogenetic organizing centers in metazoans and organizes patterning and regulates cell fate decisions. In the slug, the tip continues to function as a signaling and organizing center by generating oscillatory cAMP waves that are propagated through the slug and are responsible for cell movement in the slug (46, 47). Here we show that although FLN$^-$ strains form a tip, in mixing experiments with wild type cells, the wild type cells sort to the tip. These results and the results from rescue experiments with mutant cells expressing ddFLN under the control of a prestalk promoter *versus* a prespore promoter underline the role of filamin in morphogenesis as has been suggested from mutations in the human FLN genes (18).

The experiments presented here were mainly concerned with the rescue properties of filamin proteins in phototaxis. Two hypotheses have been proposed to explain phototactic turning (3, 5, 48). The optical hypothesis assumes that the pseudoplasmodium acts as a cylindrical lens, causing stronger stimulation of light locally and speeding up cell movement in the tip, thus leading to bending of the anterior zone toward the light source. The signal transduction or sign reversal model assumes that light acts directly on physiological processes of the cell and on cell-cell signaling, thereby shifting the position of the organizing center in the tip. Both hypotheses require a "sensor," which acts as a photoreceptor. Searching the *Dictyostelium* genome did not result in the identification of obvious candidates for a photoreceptor, a cryptochrome, or phytochrome or light sensory domains, although numerous genes coding for seven transmembrane receptors or phytochromes that are histidine kinases are present (49).

Our data suggest that F-actin cross-linking by filamin is essential for phototactic turning of *Dictyostelium* slugs because shortened filamin molecules containing different numbers of repeats that bind to but no longer cross-link actin filaments do not lead to a reversal of the phototaxis defect. The importance of F-actin cross-linking was also recognized during the analysis of *Drosophila* oogenesis (20). In *Drosophila* oogenesis filamin is involved in early follicle cell morphogenesis. Follicle cells are important for germline cyst encapsulation. Filamin alleles leading to the expression of C-terminally truncated forms still allowed cyst encapsulation and egg chamber formation while interfering with ring canal assembly. One can assume two roles for filamin in these processes; full-length filamin allows recruitment of filamentous actin to germline ring canals and the maintenance of the cortical actin cytoskeleton in nurse cells, whereas the truncated forms are sufficient for early follicle cell migration. It was proposed that the latter molecules generate a mechanically stable cortex required for the shape changes and cell contact formation during migration. Similarly, slug migration is improved when filamin molecules are present in the cell cortex. This might allow the cells in the slug to perform interactions with the surrounding cells and undergo cell shape changes as they migrate.

Filamins lacking internal repeats, FLNΔrod2-GFP and FLNΔrod4-GFP, do not rescue the phototaxis defect either. Because they can still dimerize through their C termini, they should be able to cross-link actin filaments. The lack of individual repeats could, however, influence the geometry of the network formed. This might be particularly relevant for FLNΔrod4-GFP, which is lacking the rod repeat distinguished by a unique unfolding and refolding behavior. Repeat 4 unfolds easiest and has a stable folding intermediate. In the first stage of unfolding about 40 residues corresponding to the first two β-strands are unfolded from the molecule, whereas the remaining 60 residues form a folded intermediate (33). The intermediate is a stable structure able to fold upon itself. The two-stage folding of the fourth repeat is also the fastest process of folding observed for the rod domain (34). The biological significance of this feature might be that this repeat would allow for the whole rod to nearly double its length and then come back to its native state. It is also possible that the folding speed of an elongated molecule is increased by the presence of the intermediate as the free energy barrier between the unfolded state and the intermediate, and in the next step, the intermediate and a fully folded protein is easier to break than for direct transition to the folded state. The presence of repeat 4 in FLNΔrod2-GFP did, however, not improve its ability to restore phototaxis. These results do not exclude a regulatory role for the rod by providing a platform for interacting proteins. Bound or released interacting molecules may be part of signaling pathways that are involved in phototactic signaling.

The failure of *Dictyostelium* FLN$^-$ cells in phototaxis and tip cell formation and the rescue activity of full-length FLN point directly to an involvement of the protein in signaling. Thus it might well be that it is crucially involved in the generation of a cytoskeletal network that allows the formation of signaling complexes near the membranes or that it functions as a sensor molecule as described above. Investigations by Dormann and Weijer (46) have provided compelling evidence that phototactic movement of the slug is connected to cAMP signaling, and one possibility is that cAMP signaling during the slug stage is disturbed in the filamin minus mutant because of altered expression of the components of the cAMP signaling system (reviewed in Ref. 47). This is supported by a microarray analysis where we compared the gene expression in wild type and FLN$^-$ cells that indicated that genes regulating cAMP levels and cell-cell adhesion molecules are altered in their expression (data not shown). The down-regulation of the cAMP phosphodiesterase gene (PDE), which is normally expressed in prestalk cells as a result of the activity of a specific promoter (50), and the up-regulation of its inhibitor, the glycoprotein phosphodiesterase inhibitor, could cause elevated levels of cAMP. An increased extracellular cAMP concentration in the mutant might interfere with the formation of the three-dimensional scroll wave by increasing the length of the adaptation and thus the period of wave propagation. Generation of a PDE mutant pointed out the essential role of PDE in slug morphogenesis. Its role is also supported by results obtained from experiments in which phosphodiesterase inhibitor expression from a prestalk-specific promoter inhibited PDE activity and blocked slug morphogenesis (51).

The role of ddFLN in phototactic migration is probably complex. From our results we conclude that the protein functions as an F-actin cross-linking protein and is also involved in signaling, which controls the slug migration at the multicellular stage of *Dictyostelium* development. Expression of the full-length protein in prestalk cells is essential and sufficient for a complete rescue. In the tip it may be necessary for propagation of the scroll wave, which controls slug migration. Filamin might also support the formation of a dynamic structure surrounding the organization center, and its loss in this region may cause bimodal phototaxis.

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