Chemoattractant-controlled accumulation of coronin at the leading edge of *Dictyostelium* cells monitored using a green fluorescent protein–coronin fusion protein

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Background: The highly motile cells of *Dictyostelium discoideum* rapidly remodel their actin filament system when they change their direction of locomotion either spontaneously or in response to chemoattractant. Coronin is a cytoplasmic actin-associated protein that accumulates at the cortical sites of moving cells and contributes to the dynamics of the actin system. It is a member of the WD-repeat family of proteins and is known to interact with actin–myosin complexes. In coronin null mutants, cell locomotion is slowed down and cytokinesis is impaired.

Results: We have visualized the redistribution of coronin by fluorescence imaging of motile cells that have been transfected with an expression plasmid containing the coding sequence of coronin fused to the sequence encoding the green fluorescent protein (GFP). This coronin–GFP fusion protein transiently accumulates in the front regions of growth-phase cells, reflecting the changing positions of leading edges and the competition between them. During the aggregation stage, local accumulation of coronin–GFP is biased by chemotactic orientation of the cells in gradients of cAMP. The impairment of cell motility in coronin null mutants shows that coronin has an important function at the front region of the cells. The mutant cells are distinguished by the formation of extended particle-free zones at their front regions, from where pseudopods often break out as blebs. Cytochalasin A reduces the size of these zones, indicating that actin filaments prevent entry of the particles.

Conclusions: These data demonstrate that coronin is reversibly recruited from the cytoplasm and is incorporated into the actin network of a nascent leading edge, where it participates in the reorganization of the cytoskeleton. Monitoring the dynamics of protein assembly using GFP fusion proteins and fluorescence microscopy promises to be a generally applicable method for studying the dynamics of cytoskeletal proteins in moving and dividing cells.

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Background

Amoeboid cells continuously remodel their motility systems during locomotion, either as a result of internal controls or in response to external signals. Extensive remodeling of the actin network accompanies the extension of pseudopods at the front of a cell. Remodeling also occurs during cytokinesis, as shown, for instance, by the spatial separation of myosin II and the actin-associated protein coronin — myosin assembles in the cleavage furrow [1] and coronin accumulates at the fronts of the emerging daughter cells [2,3].

In aggregating *Dictyostelium* cells, it is evident that the sites of leading-edge formation are not fixed by any stable structure. After local application of the chemoattractant cAMP, it takes only seconds for a front to become a tail and *vice versa* [3,4]. Remodeling of the actin system in a moving *Dictyostelium* cell implies that certain proteins are sorted to specific sites in pseudopods [5,6]. For instance, talin is recruited in response to chemoattractant from the cytoplasm and assembles, together with actin filaments, into an ordered fabric that becomes the core of an incipient leading edge [7]. Coronin is another protein that is associated with the acto-myosin system *in vitro* and *in vivo* [8]. The finding that coronin null mutant cells move only half as fast as wild-type cells has demonstrated the importance of coronin in cell locomotion [2].

Results

In order to provide a stable and visible tag for coronin in living cells, we constructed an expression vector encoding a fusion protein in which the green fluorescent protein (GFP) of *Aequorea victoria* is attached to the carboxyl terminus of *D. discoideum* coronin (Fig. 1; [9,10]). For comparison, we also expressed the GFP protein alone in *D. discoideum* cells. In both types of transformants, we detected brilliant green fluorescence. Because the GFP protein was uniformly distributed in the cytoplasm, variations in fluorescence intensity could be used to determine the thickness profile along the body of moving cells.

The distribution of fluorescence intensity of the coronin–GFP fusion protein differed from that of free GFP. In growth-phase cells — undeveloped cells that tend to extend multiple fronts during locomotion — coronin–GFP was continuously reshuttled between the cytoplasm and the actual front regions (Fig. 2). As accumulation at

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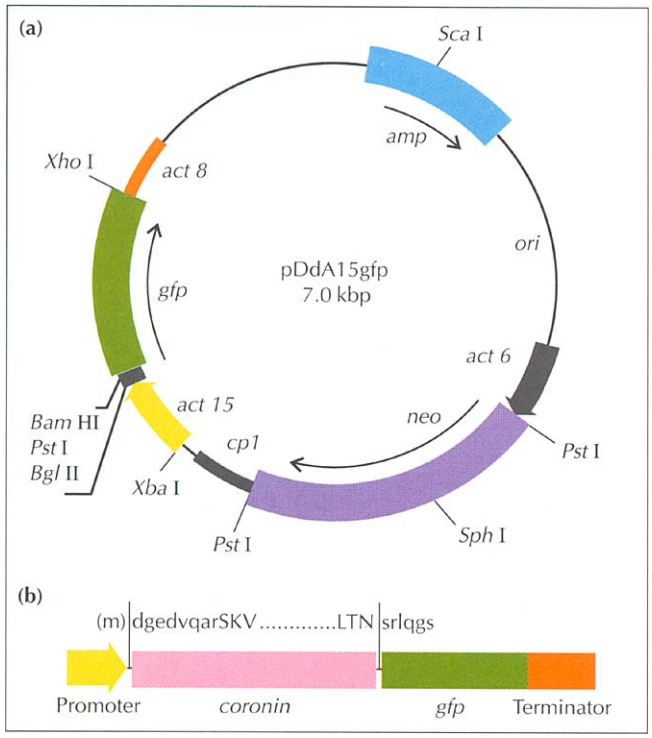

Fig. 1. Constructs used for the expression of coronin–GFP. **(a)** The *actin 15* promoter (*act 15*) was inserted into vector pDdgfp (which was originally used to express GFP in *D. discoideum*), creating plasmid pDdA15gfp. In addition to the *gfp* cassette, which was preceded by a multiple cloning site, the vector contained a neomycin-resistance cassette (*neo*) for the selection of transformants in *D. discoideum* and an ampicillin-resistance cassette (*amp*) for selection in *Escherichia coli*. **(b)** The coronin coding region was cloned in front of the *gfp* sequence of pDdA15gfp. The relevant portion of the resulting plasmid, pDdA15cor–gfp, is shown here. Transcription of the gene encoding the fusion protein was controlled by the *act 15* promoter and the *act 8* terminator (*act 8*) from *D. discoideum*. The coronin–GFP fusion protein produced by the vector pDdA15cor–gfp contained 8 amino-terminal amino acids (behind the initial methionine) encoded by *act 15* (lower case; single-letter amino-acid code), followed by the 444 amino acids of coronin (upper case). The coronin and GFP sequences were separated by six amino acids encoded by the polylinker (lower case). '*act 6*' refers to the *actin 6* promoter; '*cp1*' refers to the *cp1* terminator.

the front regions occurred in the absence of an extraneous signal, it should be the result of internal processes that make the leading edges distinct from other regions of the actin cortex.

Aggregation-competent cells acquire a cylindrical shape with a well defined front and tail. In these cells, coronin accumulates to high levels at the leading edge, as previously shown by immunofluorescence labeling [8]. The polarity of these cells can be altered by the local application, with a micropipette, of the chemoattractant cAMP, which is capable of eliciting a new leading edge at any region of the cell surface. By using the coronin–GFP fusion protein, we were able to visualize, with high temporal resolution, the redistribution of coronin that follows stimulation of the cells with cAMP.

Results from an experiment in which a coronin–GFP-containing cell repeatedly reoriented in response to changing positions of the micropipette are illustrated in Figure 3b. The cell showed local accumulation of the fusion protein in a variety of situations. Competition between the initial front and a chemoattractant-induced lateral front was reflected in redistribution of the fusion protein (frames 15–60). For a few seconds both fronts intensely fluoresced (frames 40,45) before the lateral front took over the function of a leading edge (frame 60). Upon repositioning the micropipette from top to bottom (frame 105), the cell turned in the direction of the new gradient, while retaining its established front (frames 145, 175). This reorientation was again accompanied by the accumulation of coronin–GFP at the leading edge (frame 175). The final sequence in Figure 3b (frames 175–305) shows the conversion of the tail region of a cell into a front. During this reversal of polarity, the intensity of fluorescence decreased at the previous front and increased at the new one. As a control, cells producing only GFP were recorded (Fig. 3a).

Analysis of a number of chemoattractant-stimulated cells revealed variations in the temporal relationship between

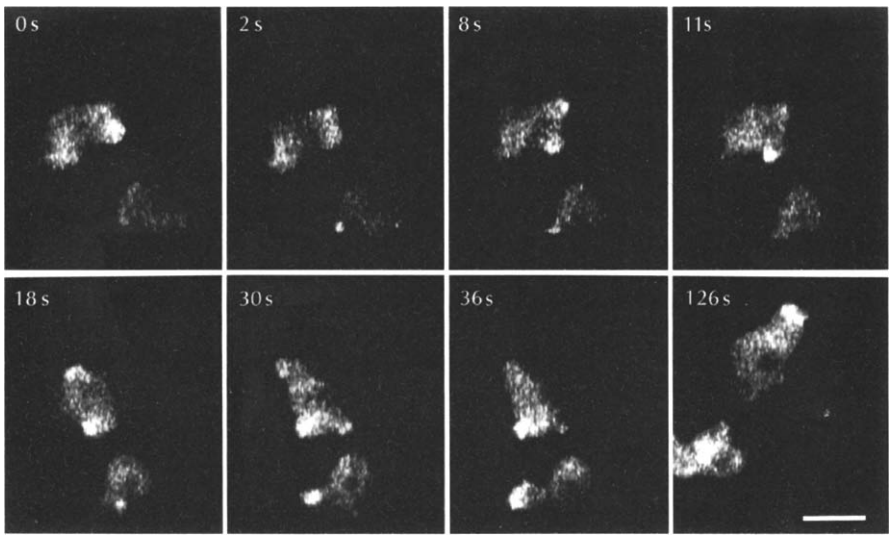

Fig. 2. Dynamics of coronin–GFP redistribution during locomotion of a *D. discoideum* growth-phase cell. The fluorescence images of the cell moving on glass show the formation of multiple pseudopodia, as typical of that stage, accompanied by accumulation of the fusion protein at the actual front regions. Scale bar = 10 μm.

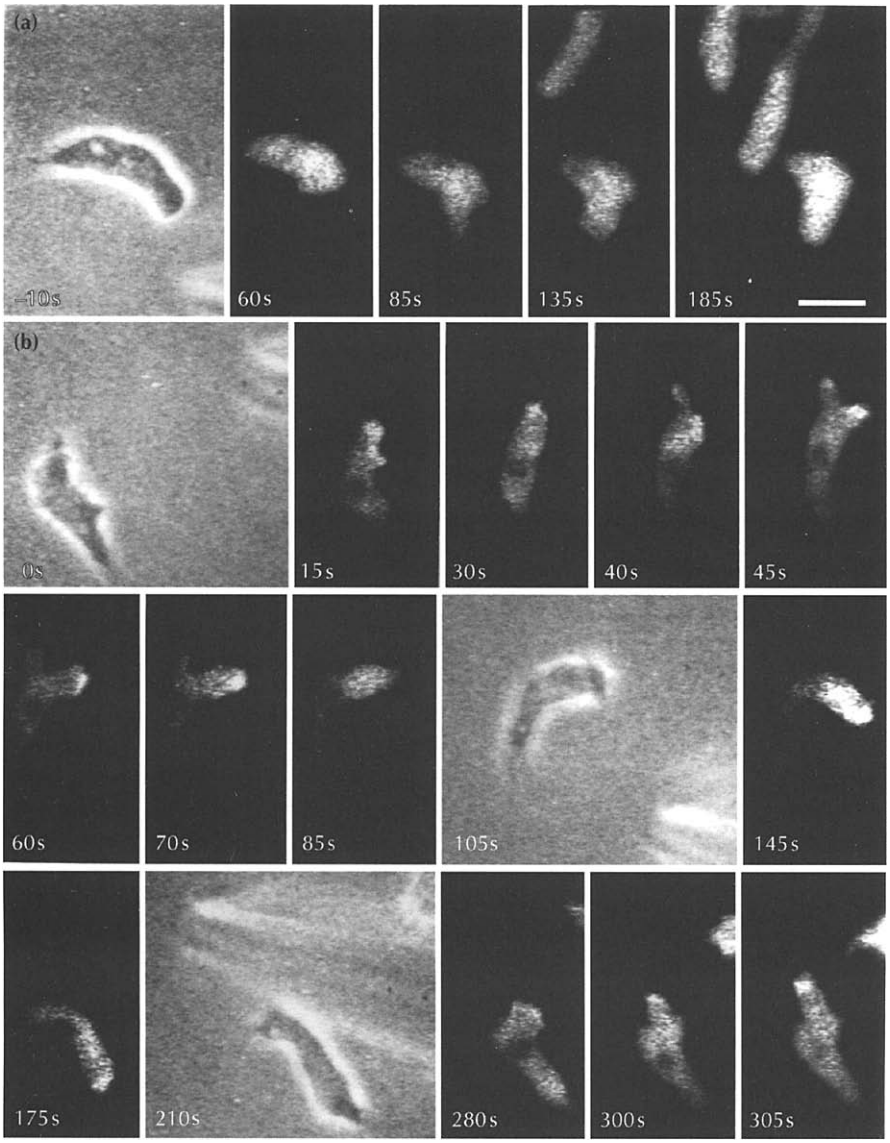

Fig. 3. **(a)** Distribution of GFP in the cytoplasm, and **(b)** accumulation of coronin–GFP at the front region of chemoattractant-stimulated cells. Cells at the aggregation-competent stage were typically elongated and capable of changing their polarity in response to gradients of cAMP. Numbers indicate seconds before or after implantation of a micropipette filled with cAMP. The phase-contrast images show cell shape and position of the micropipette, fluorescence images show the distribution of GFP (a) or coronin–GFP (b). In (a) one cell extends a new pseudopod towards the micropipette at the lower right corner; two other cells are attracted from a larger distance. None of the cells shows an accumulation of GFP at the front region. In (b) a cell that repeatedly reoriented in response to changing positions of the micropipette shows transient accumulation of coronin–GFP at its alternating front regions. The nucleus is recognized as a dark area. Positions of the micropipette were changed at 100 sec and 205 sec. Scale bar = 10 μm.

the appearance of a new front and the first sign of coronin–GFP accumulation at the site of this front. On average, the local accumulation of coronin–GFP was seen 7 seconds after a protrusion became detectable, but coronin–GFP accumulation could also precede the protrusion of a front by 5 seconds. Later, during chemotactic movement, the phases of obvious coronin–GFP accumulation were sometimes interrupted by phases in which fluorescence did not appear to be enhanced at the front. In Figure 3b these intermittent phases are represented by frame 85. The impression, from visually analyzing this and other recordings, is that the accumulation of coronin–GFP at the front of a cell is most pronounced during phases of fast and precisely oriented locomotion.

To exclude artifacts caused by the GFP domain of the fusion protein, untransformed cells, which contain only normal coronin, were locally stimulated with cAMP. After the extension of a new front, at 1 minute of stimulation, the cells were fixed (as previously reported for talin [7]) and labelled with a monoclonal antibody specific for coronin, mAb 176-3-6, which was visualized with a fluorescent second antibody. The immunofluorescence was at its most intense at a newly induced pseudopod (data not shown). This method has the disadvantage that coronin redistribution is visualized only at the time of fixation. The data complement the results obtained using coronin–GFP, however, by demonstrating that it is the coronin moiety of the fusion protein which is responsible for chemoattractant-induced relocalization.

The spontaneous and cAMP-receptor-mediated redistribution of coronin suggests that it has a function in the cytoskeleton close to the front of a cell. Direct evidence for such a function in the control of the organization and dynamics of the motility system was provided by the analysis of a coronin null mutant. Imaging of the mutant cells by phase-contrast optics revealed enlarged zones along their borderlines that were free of organelles, and therefore appeared as hyaline areas (Figs 4,5). In growth-phase cells or in unstimulated aggregation-competent cells, which are irregular in shape, the hyaline areas were divided into separate zones, which coincided with the multiple leading edges of these cells. Upon chemotactic

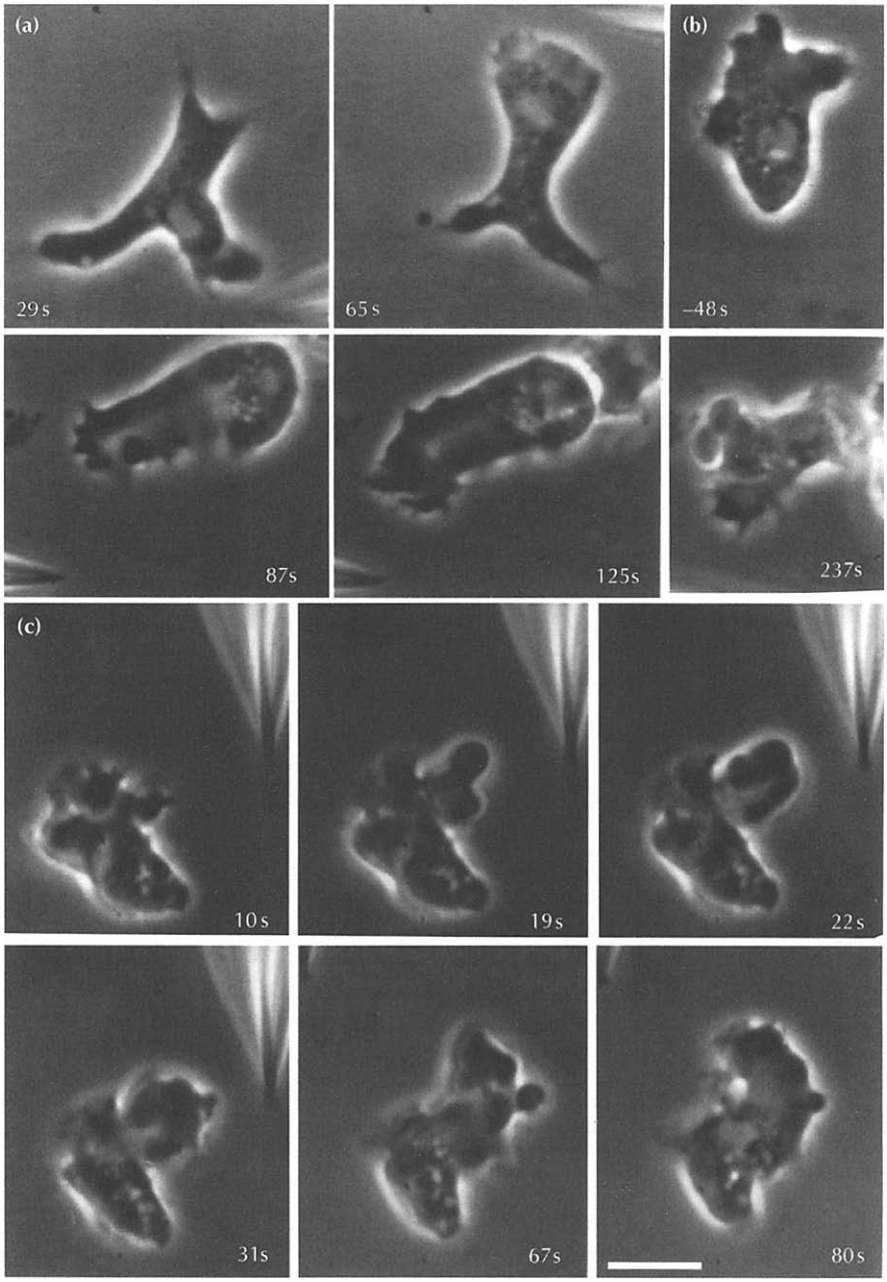

Fig. 4. Chemotactic responses of a wild-type cell **(a)** and of two coronin null cells **(b,c)**, visualized by phase-contrast optics. Numbers indicate seconds before or after insertion of a micropipette. (a) A small hyaline rim is seen at the leading edges that have been induced at two sites on the surface of the wild-type cell by cAMP gradients running in different directions. (b) More than half of the cell area is occupied by a hyaline zone during attraction of the coronin null mutant cell by cAMP (87 sec and 125 sec). After removal of the pipette, extensive blebbing is seen (c). A coronin null mutant cell responding to the attractant by extending pseudopods in form of blebs (19 sec), which subsequently fuse together into a large lobopodium covered by rounded surface extensions (67 sec). Scale bar = 10 μm.

stimulation, the mutant cells became elongated, as did the wild-type cells. At the same time, the hyaline areas of the mutant cells became more evident because they were concentrated at the front regions of these cells (Fig. 4).These areas sometimes occupied more than half of the total cell area, causing mitochondria, vesicles and nuclei to be confined to the rear of the mutant cells.

That the actin system is altered in coronin null mutant cells is suggested by the effect on them of cytochalasin A. Within a narrow range of concentrations, this actin-depolymerizing agent reduced the size of the hyaline area in the mutant cells to a size similar to that observed in the wild-type cells (Fig. 6). Within 15 minutes of incubation in 4 μM cytochalasin A, the hyaline zones became indistinct as vesicles, mitochondria and other particles entered them. The mutant cells rounded up only slowly: after 45 minutes at this drug concentration, most of the mutant cells remained spread, exhibited cytoplasmic streaming and had tapering pseudopods; after 60 minutes, about half of the cells had rounded up. At higher concentrations of cytochalasin A, both coronin null mutant and wild-type cells were immobilized.

Discussion

Coronin associates with actin–myosin complexes [8], and the phenotypic changes observed in coronin null mutant cells are most likely due to a change in the actin network that prevents the movement of particles into the front region of a cell. The slowness of coronin null mutant cells suggests that coronin speeds up the disassembly of the actin network according to the requirements of

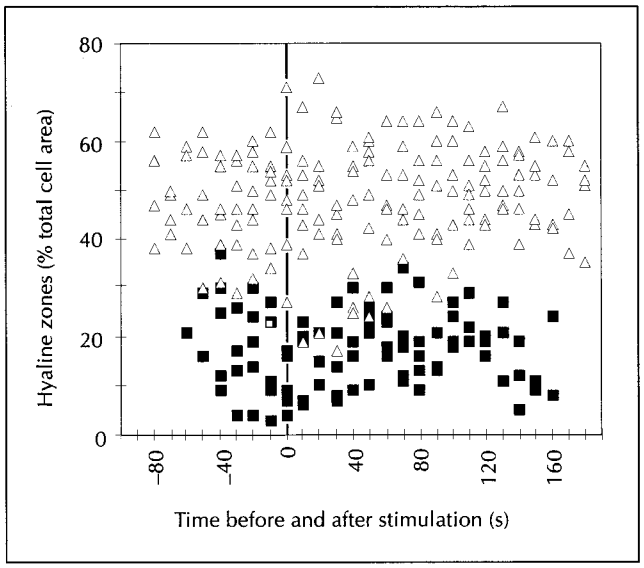

Fig. 5. Sizes of hyaline areas in wild-type (closed symbols) and coronin null cells (open symbols). Cells were chemotactically stimulated with cAMP administered through a micropipette at time zero. Symbols represent single cell measurements.

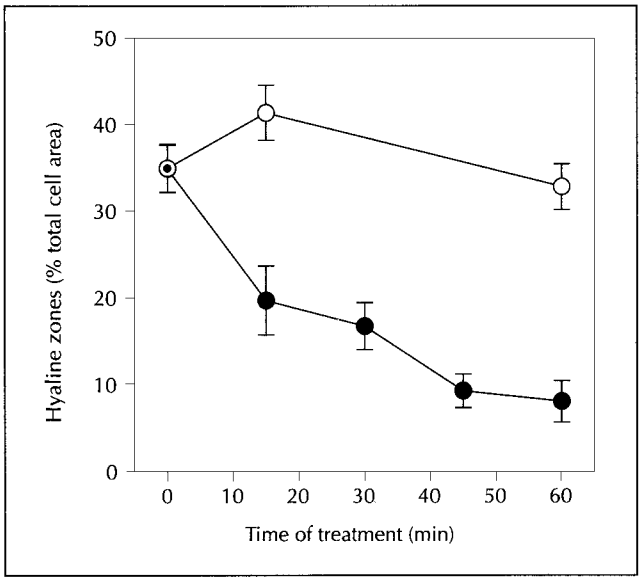

Fig. 6. Reduction in the sizes of hyaline areas in coronin null mutant cells in response to cytochalasin A. Control cells treated with 0.2 % dimethylsulphoxide (DMSO) in Na^+/K^+-phosphate buffer, pH 6.0 (○). Cells treated with 4 μM cytochalasin A in the same solution (●). Data represent between 9 and 18 measurements on single aggregation-competent cells; the bars indicate standard errors.

rapidly moving cells. The partial rescue to a wild-type cell-organization phenotype by treatment of coronin null mutant cells with cytochalasin A, suggests that — in *Dictyostelium* cells, as well as in neutrophils [11,12] — coronin balances the activities of proteins that nucleate actin polymerization and thus, together with crosslinking proteins, tends to stabilize the network of actin filaments in the cell cortex. In this context, it is of interest that a coronin-like protein has been cloned from a human granulocyte cell line [13].

Coronin most likely cooperates with other proteins in helping to restructure the actin skeleton during cell locomotion and chemotactic orientation. According to its sequence, coronin is a member of the WD-repeat family of proteins [14]. These proteins contain a conserved core structure capable of undergoing pairwise or multimeric interactions. The core is thought to provide a scaffold for the display of variable regions on the surface of the molecules [14]. The best known members of WD-repeat proteins — the β-subunits of heterotrimeric G proteins — act in signal transduction by forming multiprotein complexes. The amino-terminal domain of coronin contains five WD repeats [8]. It is likely, therefore, that coronin binds not only to actin but also to other proteins. In this way it may couple regulatory proteins to the actin–myosin system responsible for cell locomotion. The recent discovery of a coronin homologue expressed in the human immune system and brain cells [13] suggests members of the coronin subfamily of WD-repeat proteins fulfil a general function in motility and shape changes in eukaryotic cells.

From a technical point of view, our results show that the dynamics of relocalization of a cytoskeletal protein can be visualized in real time within moving cells by the use of a GFP tag. Extending this method to the study of other proteins that regulate the actin system may provide a comprehensive overview of the distribution and dynamics of actin-associated proteins during cell locomotion. Distinct local activities are required for the protrusion and retraction of the cell body, and for its attachment to, and detachment from, the substratum [15]. The persistence of direction in a moving cell depends on interactions in the cytoskeleton that coordinate these activities, so that the stages of locomotion are linked together in spatio-temporal patterns [16]. An important question is to which extent sorting of proteins contributes to these local activities and to their coordination in a moving cell.

Materials and methods

Cell culture

Cells of wild-type strain AX2-214, of the coronin null mutant HG1569 [2], or of transformants were cultivated axenically in shaken suspension up to not more than 5×10^6 cells per ml. To induce development to the aggregation-competent stage, cells were starved for 6 h at a density of 1×10^7 cells per ml in 17 mM Na^+/K^+-phosphate buffer, pH 6.0, as described [17].

Vector construction and cell transformation

The vector pDdA15gfp (Fig. 1a) was derived from the pDdGal-15 plasmid [18]. The lacZ gene of pDdGal-15 was replaced with a 741 base-pair *BamHI–XhoI* fragment encompassing the entire *gfp* coding region from the construct pVT-Ugfp. The *gfp* fragment in this construct was amplified by the polymerase chain reaction (PCR) and contained *BamHI* and *SacI* sites at its 5′ and 3′ ends, respectively. In addition, the construct contained the *act 15* promoter region of *D. discoideum* [19] cloned into the *XbaI* and *BglII* sites, in frame with the *gfp* sequence.

The *coronin* cDNA was amplified by PCR and inserted into the *Bgl*II site of pDdA15gfp. The relevant portion of vector pDdA15cor–gfp is shown in Figure 1b. *D. discoideum* AX2 cells were transformed with either pDdA15gfp or pDdA15cor–gfp using the calcium phosphate method [20]. To isolate coronin–GFP-expressing cells, G418-resistant transformants were cloned on SM nutrient agar plates with *Klebsiella aerogenes*. The clones were analyzed by immunoblotting with an anti-coronin monoclonal antibody, mAb 176-3-6 [2]. The immunoblotting experiments revealed that the relevant clones contained, in addition to the endogenous coronin, a labelled protein corresponding to the size of the fusion protein. The band corresponding to this protein showed green fluorescence when unheated samples were run on SDS–polyacrylamide gels and were viewed on a UV transilluminator.

Chemotactic stimulation through micropipettes and image analysis

Cells were washed and diluted in the phosphate buffer before use and transferred onto a glass surface. Starved, aggregation-competent cells were stimulated using micropipettes [21] filled with 1×10^{-4} M cAMP. Cell movement was recorded on a Panasonic AG-6720A-E video recorder using a Zeiss IM 35 inverted microscope equipped with a water condenser and a phase-contrast 100 × oil plan neofluar objective lens. GFP fluorescence images were taken with a Zeiss Axiovert 135 TV microscope with an HBO 50 mercury lamp and an 100 × oil neofluar objective lens in combination with a SIT camera C2400-08 (Hamamatsu). The filters HQ 450/50 (exciter), Q 480 LP (beam splitter), and HQ 510/50 (emitter) were purchased from AHF Analysentechnik, Tübingen, Germany. To avoid injury to the cells by incident light, which leads to blebbing and rounding up of the cells, the light intensity was reduced to 1 % by grey filters. Hyaline and total cell areas were measured by drawing lines around these areas and counting the pixels enclosed by the lines using a PC program.

Labelling with antibodies

To follow cells that chemotactically responded to cAMP application by video recording, and to identify them after fixation, we used a CELLOCATE coverslip with grid (Eppendorf, Hamburg, Germany) as a substratum. The cells were fixed with picric acid/formaldehyde, pH 6, post-fixed with 70 % ethanol [22], and labelled with mAb 176-3-6, followed by tetramethylrhodamine isothiocyanate-conjugated goat anti-mouse IgG antibody (Jackson Immuno Research, West Grove, Pennsylvania, USA).

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