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Interaction of a *Dictyostelium* Member of the Plastin/Fimbrin Family with Actin Filaments and Actin-Myosin Complexes

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A protein purified from cytoskeletal fractions of *Dictyostelium discoideum* proved to be a member of the fimbrin/plastin family of actin-bundling proteins. Like other family members, this Ca^{2+}-inhibited 67-kDa protein contains two EF hands followed by two actin-binding sites of the α-actinin/β-spectrin type. Dd plastin interacted selectively with actin isoforms: it bound to *D. discoideum* actin and to β/γ-actin from bovine spleen but not to α-actin from rabbit skeletal muscle. Immunofluorescence labeling of growth phase cells showed accumulation of Dd plastin in cortical structures associated with cell surface extensions. In the elongated, streaming cells of the early aggregation stage, Dd plastin was enriched in the front regions. To examine how the bundled actin filaments behave in myosin II-driven motility, complexes of F-actin and Dd plastin were bound to immobilized heavy meromyosin, and motility was started by photoactivating caged ATP. Actin filaments were immediately propelled out of bundles or even larger aggregates and moved on the myosin as separate filaments. This result shows that myosin can disperse an actin network when it acts as a motor and sheds light on the dynamics of protein-protein interactions in the cortex of a motile cell where myosin II and Dd plastin are simultaneously present.

INTRODUCTION

Locomotion of eukaryotic cells, cytokinesis, determination of cell shape, and phagocytosis require rapid rearrangements of the actin network. The highly motile amoebae of *Dictyostelium discoideum* are particularly suitable to study actin-binding proteins (ABPs) that regulate these rearrangements. A variety of F-actin cross-linking and bundling proteins of these cells cause microfilaments to assemble into networks or parallel bundles. The most prominent cross-linking proteins in *D. discoideum* are α-actinin and 120-kDa gelation factor (ABP120) (Brier *et al.*, 1983; Noegel *et al.*, 1989). In addition, five actin-bundling proteins have been purified from *D. discoideum* cells: two 30-kDa proteins, p30a and p30b, that differ in Ca^{2+} regulation (Fechheimer and Taylor, 1984; Brown, 1985), a 50-kDa Ca^{2+}-independent bundling protein identified as elongation factor 1α (Demma *et al.*, 1990), and two isoforms of cortexillin, proteins that are important for the cells to maintain their shape and undergo cytokinesis (Faix *et al.*, 1996).

In vitro studies and mutational analysis have suggested that any of these actin-binding proteins may contribute to cell migration, chemotactic reorientation, and phagocytosis in *D. discoideum*, but none of them has been shown to be essential. Under optimal conditions, cells lacking α-actinin and ABP120 are still capable of locomotion and chemotactic orientation (Brink *et al.*, 1990; Gerisch *et al.*, 1993). The degree to which these proteins are able to maintain cell functions in the absence of the two major F-actin cross-linkers depends not only on culture and assay conditions, it also varies among *D. discoideum* strains (Wallraff and Gerisch, unpublished observations). Lack of ABP120 alone has been reported to cause apparent alterations in cell shape and motility (Cox *et al.*, 1992, 1995). According to other reports, special

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conditions are required to detect substantial deficiencies even when both α-actinin and ABP120 are missing (Schindl *et al.*, 1995; Weber *et al.*, 1995). In the case of cortexillins, both isoforms have to be eliminated to produce dramatic phenotypic changes (Faix *et al.*, 1996). In the light of these results, it is important to identify all of the proteins involved in cell locomotion or stability of the actin skeleton and to study their contribution to cell behavior in wild-type and mutants that lack different sets of actin-binding proteins.

In this article, we characterize a member of the fimbrin/plastin family of actin-bundling proteins isolated from *D. discoideum* and study its effect on actin-myosin interaction in an in vitro motility system. The first fimbrin was identified as a major component of the microvilli in the intestinal brush border (Bretscher and Weber, 1980; Bretscher, 1981; Glenney *et al.*, 1981). The sequence of chicken fimbrin (de Arruda *et al.*, 1990) shows a high degree of homology to human L-plastin, a protein phosphorylated in response to growth factors interleukin 1 and interleukin 2 and to phorbol esters (Goldstein *et al.*, 1985; Matsushima *et al.*, 1988). In addition to L-plastin, two isoforms, T-plastin and I-plastin, have been identified in man (Lin *et al.*, 1988, 1994). In *Saccharomyces cerevisiae*, a dominant suppressor (*sac6*) of a mutation in the actin gene turned out to be a homologue of fimbrin (Drubin *et al.*, 1988; Adams *et al.*, 1989, 1991).

MATERIALS AND METHODS

Cell Culture, Antibody Production, and Immunofluorescence Labeling

Cells of *D. discoideum* strain AX2-214 were cultivated axenically in shaken suspension at 23°C and starved in 17 mM sodium/potassium phosphate buffer (pH 6.0) as described elsewhere (Brink *et al.*, 1990).

Polyclonal antibodies against p67 were raised in a rabbit using Freund's adjuvant and were affinity purified on p67 coupled to a *N*-hydroxysuccinimide-Hitrap column (Pharmacia, Uppsala, Sweden). Bound antibodies were eluted with 100 mM glycine (pH 3.0) and subsequently with 100 mM triethanolamine (pH 11.5), pooled, and used for iodination or immunofluorescence labeling. mAbs were raised in BALB/c mice by i.p. injections of p67 with and without Alugel S (Serva, Heidelberg, Germany) as an adjuvant and PAIB3Ag8-myeloma cells for hybridoma production. None of the monoclonal or polyclonal antibodies cross-reacted with any protein from 3T3 fibroblasts or mouse B-lymphocytes.

For immunofluorescence labeling, growth phase or aggregation-competent cells harvested after 6 h of starvation were allowed to move on coverslips for 30 min before fixation with methanol for 10 min at −20°C or at room temperature with picric acid/formaldehyde (15% (vol/vol) of a saturated aqueous solution of picric acid/2% paraformaldehyde, pH 6.0) followed by 70% ethanol and processing of the specimens according to Humbel and Biegelmann (1992). After washing, the fixed cells were incubated with affinity-purified polyclonal antibodies (50 μg IgG/ml) or with hybridoma culture supernatant containing mAb 210–183-1, and subsequently with tetramethylrhodamine isothiocyanate (TRITC)-conjugated goat anti-rabbit IgG or goat anti-mouse IgG (Jackson ImmunoResearch Labs, West Grove, PA). Confocal images were obtained using an LSM 410 microscope (Zeiss, Welwyn Garden City, Herts, United Kingdom).

Purification of a 67-kDa Protein (p67) from a Contracted Actin-Myosin Complex

Contracted actin-myosin complex was prepared from cells starved for 12 h essentially as described previously (Condeelis and Taylor, 1977; Fechheimer and Taylor, 1984; de Hostos *et al.*, 1991). Washed cells were resuspended at 4°C in an equal volume of homogenization buffer [5 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES), pH 6.8, 5 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, protease inhibitor cocktail (PIC) consisting of leupeptin, bestatin, pepstatin A, antipain, 10 μg/ml each] and disrupted by nitrogen decompression in a Parr bomb (800 psi), followed by gentle agitation with a loose fitting Dounce homogenizer. Particles were removed at 10,000 × *g* and at 100,000 × *g*, and contraction was induced by adding 5 mM $MgCl_2$, 20 mM KCl, 1 mM ATP, and raising the pH to 7.5. After 1 h on ice, the contracted actin-myosin gel was washed twice with homogenization buffer (pH 7.5) supplemented with $MgCl_2$, KCl, and ATP, resuspended, and dialyzed against HEPES-G buffer [5 mM HEPES, pH 7.5, 0.2 mM dithiothreitol (DTT), 0.2 mM $CaCl_2$, 0.2 mM ATP, 0.2 mM $MgCl_2$, PIC (1 μg/ml of each inhibitor), 1 mM benzamidine]. Upon fractionation of the dissolved proteins on a DE52 column, p67 eluted in a linear gradient at 75–100 mM NaCl, overlapping with coronin (de Hostos *et al.*, 1991) and a 54-kDa polypeptide, which was most likely a degradation product. Its amount could be reduced by minimizing proteolysis. (A polypeptide of similar size was produced by digesting p67 with papain.)

Coronin and the 54-kDa polypeptide were removed from p67 by binding to a Mono S column in 25 mM 2-(*N*-morpholino)ethanesulfonic acid buffer (pH 6.0) with 0.2 mM DTT and elution with a linear 0–250 mM NaCl gradient. The yield was 100–200 μg of p67 from 5×10^{10} cells, which corresponds to about 80 g of wet weight.

Purification of p67 from the Detergent-insoluble Cytoskeleton

For large-scale preparation of p67, growth phase cells were washed and resuspended in two volumes of lysis buffer [80 mM PIPES, pH 6.8, 30% glycerol, 0.5 mM DTT, 5 mM EGTA, 5 mM $MgCl_2$, 1 mM phenylmethylsulfonyl fluoride, PIC (10 μg/ml of each inhibitor)] and lysed with 1% Triton X-100 for 5 min at room temperature. All subsequent steps were done at 4°C on ice. Microfilaments, microtubules, and associated proteins were pelleted for 3 min at 14,000 × *g* and washed twice with lysis buffer without detergent. The pellet was extracted for 1 h in Ca^{2+}-containing buffer [50 mM PIPES, pH 7.0, 0.5 mM DTT, 1 mM $MgCl_2$, 20 mM $CaCl_2$, PIC (10 μg/ml of each inhibitor)]. The extract was centrifuged for 20 min at 130,000 × *g*, and the supernatant was applied to a DE52 column. p67 was recovered in the flow-through, whereas coextracted actin and α-actinin remained bound to the column. The flow-through was dialyzed against buffer consisting of 20 mM Tris (pH 8.0), 0.2 mM DTT, 0.1 mM $CaCl_2$, and PIC (1 μg/ml of each inhibitor), subjected to a Mono Q column, and eluted in a linear gradient at 150–170 mM NaCl. The protein was purified to homogeneity on a Mono S column in 25 mM 2-(*N*-morpholino)ethanesulfonic acid buffer (pH 6.0) containing 0.2 mM DTT. In a linear gradient of NaCl, p67 eluted at 75–100 mM. The yield was 2–4 mg p67 from 5×10^{10} cells.

Immunoscreening of a cDNA Library, Cloning, and DNA Sequencing

A λgt11 expression library prepared from mRNA of 4-h starved *D. discoideum* strain AX3 cells (Clonetech Inc., Palo Alto, CA) was plated on *E. coli* RY1090 and

screened with ^{125}I-labeled affinity-purified polyclonal antibodies against p67. Two cDNA clones were digested with *Eco*RI, and the fragments were subcloned into pUC19. The cDNAs were sequenced in both orientations with a T7 polymerase sequencing kit (Pharmacia), and the sequences were analyzed using FASTA and BESTFIT of the UWGCG software (Devereux *et al.*, 1984) and the MIPSX database (Max-Planck-Institut für Biochemie, Martinsried, Germany).

Cosedimentation and Bundling Activity of Dd Plastin

Actin from *D. discoideum* AX2 cells was purified according to Spudich (1974), actin from rabbit skeletal muscle from acetone powder according to the method of Spudich and Watt (1971) with an additional gel filtration step on a S300 column, and β/γ-actin from bovine spleen according to Rozycki *et al.* (1991). Proteins were dialyzed at 4°C against 10 mM imidazole buffer (pH 7.3) containing 0.1 mM $MgCl_2$, 0.1 mM ATP, and 0.2 mM DTT, and centrifuged at 110,000 × *g* to remove aggregates. G-actin (6 μM) was polymerized in the presence or absence of Dd plastin by the addition of 10× concentrated polymerization buffer (same as dialysis buffer plus 20 mM $MgCl_2$ and 10 mM EGTA or 20 mM $MgCl_2$ and 2 mM $CaCl_2$) for 2 h at room temperature.

To determine the F-actin bundling activity of Dd plastin, the mixture was centrifuged for 30 min at 15,000 × *g* in an Eppendorf centrifuge. Binding to F-actin was determined by high-speed centrifugation for 30 min at 110,000 × *g* in an airfuge. One third of the supernatant was carefully collected from the top and mixed with 10× SDS sample buffer. Proteins in the supernatant and pellet fractions were separated by SDS-PAGE and stained with Coomassie blue for densitometry. Values for Dd plastin in the pellet were corrected for nonspecific binding with bovine serum albumin.

Light Scattering and Viscometry

Right-angle scattering of the actin samples was measured at 350 nm using cross-polarizers in an SLM-Aminco AB2 spectrophotometer. For low-shear viscometry, polymerization was started in samples containing 5 μM G-actin and varying concentrations of Dd plastin by the addition of 10× polymerization buffer with 1 mM EGTA. Measurements were taken after 20 min of incubation at 25°C in a falling ball viscometer (MacLean-Fletcher and Pollard, 1980).

In Vitro Motility Assay

The in vitro motility assay was conducted according to Kron *et al.* (1991) and Kinosita *et al.* (1991) as described by Heidecker *et al.* (1995). Ten micromolar G-actin from *D. discoideum* was polymerized with 15 μM TRITC-conjugated phalloidin (Fluka, Buchs, Switzerland) in buffer A (10 mM imidazole, pH 7.3, 1 mM EGTA, 2 mM DTT, 2 mM $MgCl_2$) with or without Dd plastin. A flow chamber was loaded with the polymerized solution diluted 1:50 in buffer B (25 mM imidazole, pH 7.3, 30 mM KCl, 1 mM DTT). Actin filaments were allowed to settle for 60 s onto the heavy meromyosin monolayer before unbound actin and Dd plastin were removed, and the chamber was filled with 1 mM caged ATP (Molecular Probes, Eugene, OR) in buffer B. Sliding of filaments was initiated by a pulse of 340–400 nm of UV light as described by Marriott and Heidecker (1996). Actin filaments were imaged using an Axiovert 35 fluorescence microscope (Zeiss) and an intensified CCD camera (Hamamatsu, Hamamatsu City, Japan).

Analytical Methods

Dd plastin concentrations were determined according to the method of Bradford using bovine serum albumin as standard. G-actin concentrations were monitored at 290 nm using an extinction coefficient of 0.65 for 1 mg ml^{-1} of actin (Wegner and Isenberg, 1983). Proteins were separated by SDS-PAGE in a 8–18% gradient or in 12% minigels, transferred to nitrocellulose, and probed with ^{125}I-labeled antibodies. For partial amino acid sequencing, Dd plastin was eluted from SDS-polyacrylamide gels and digested with Lys-C (Boehringer Mannheim, Mannheim, Germany). Fragments were separated by reversed phase high-performane liquid chromatography and microsequenced on an LF 3600 Porton gas phase sequencer (Beckman, Fullerton, CA). Total cellular RNA was isolated as described by André *et al.* (1989), separated in 1.2% agarose gels in the presence of 6% formaldehyde, and probed with ^{32}P-labeled Dd plastin cDNA. Northern and Southern blots were hybridized in 50% formamide.

RESULTS

A 67-kDa Protein (p67) that Belongs to the Fimbrin/Plastin Family of Actin-binding Proteins

Contracted actomyosin pellets prepared from the cytosol of *D. discoideum* contained, in addition to known proteins, an unknown 67-kDa polypeptide (p67) detectable after SDS-PAGE by Coomassie blue staining (Figure 1A). After dissociation of the actomyosin complex, p67 was purified and used for polyclonal and mAb production. The same protein was obtained in a much higher yield from detergent-extracted cytoskeletons by extraction with Ca^{2+} and purified to homogeneity for use in biochemical assays (Figure 1B).

cDNAs obtained by screening an expression library with polyclonal anti-p67 antibodies encoded a protein

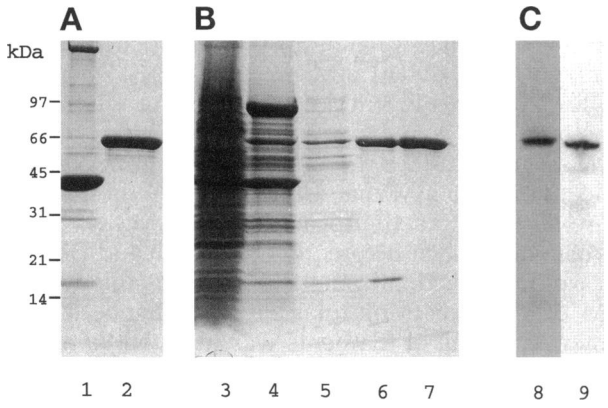

Figure 1. Purification of p67 from *D. discoideum* cells and specificity of antibodies raised against this protein. (A) Purification from a contracted actin-myosin complex. This complex contains actin and myosin II heavy chain as the most strongly stained polypeptides and p67 as a minor component (lane 1). p67 was purified by DEAE and Mono S ion exchange chromatography (lane 2). (B) Purification of p67 from a Triton X-100-insoluble cytoskeleton fraction. Lanes 3–7 show stained proteins in total cell homogenate (lane 3); proteins extracted by Ca^{2+} from the cytoskeleton fraction (the 95-kDa band corresponds to α-actinin, lane 4); flow-through at pH 7.0 from a DEAE column (lane 5); p67 containing fraction bound at pH 8.0 to a Mono Q column (lane 6); and purified p67 after Mono S chromatography (lane 7). (C) Proteins of total cell homogenate labeled with mAb 210–183-1 (lane 8) or with rabbit antiserum (lane 9). Proteins were separated by SDS-PAGE in an 8–18% gradient gel and stained by Coomassie blue in lanes 1–7 or blotted and labeled with antibodies in lanes 8 and 9.

of 610 amino acid residues, with a molecular mass of 67.0 kDa and a calculated isoelectric point of 5.96. An AT-rich region found upstream of the first ATG is typical of untranslated regions in *D. discoideum*, and in the 3′ region a polyadenylation signal overlapped with the TAA stop codon. Partial sequences of seven peptides, obtained by cleaving p67 with Lys-C endoproteinase, confirmed that the protein encoded by the cDNA is p67 (Figure 2). Southern blot analysis at high stringency indicated a single gene for p67. Although Northern blots showed an accumulation of the 2.1-kb transcript of Dd plastin during the preaggregation phase at 3–6 h of starvation, no substantial difference in the amount of the protein could be seen to accompany the changes in cell shape between growing and developing cells (not shown).

Sequence comparison shows the closest relationship of p67 to members of the fimbrin/plastin family: 48–50% identity with human T-plastin, L-plastin, and I-plastin, 48% with chicken brush border fimbrin, 45% with Sac6p from *S. cerevisiae*, but only 37% with fimbrin from *Schistosoma mansoni*. Polyclonal antibodies H222 against chicken brush border fimbrin (a gift from Dr. Anthony Bretscher) cross-reacted with p67 in immunoblots. The *Dictyostelium* sequence is slightly shorter than the sequences of other members of the fimbrin/plastin family due to two gaps: the first between residues 314 and 320, the other between residues 384 and 390 of chicken fimbrin. The first gap is located in the region of highest divergency between the human isoforms of plastin (Lin *et al.*, 1994) and is also found in the yeast sequence. The second gap is in the region that separates the two actin-binding domains and is also found in the *Schistosoma* sequence. Because of its close relationship to mammalian plastins, we refer to p67 from *D. discoideum* as Dd plastin.

Like its homologues, Dd plastin consists of four modules: a pair of EF hand motifs in the N-terminal region and two actin-binding domains of about 250 amino acid residues: one in the center and the other in the C-terminal region. The sequences of these domains show 22–31% identity to the actin-binding domains of α-actinin, β-spectrin, and dystrophin, and both domains contain a highly conserved motif of 27 amino acids, which is presumed to be essential for F-actin binding (Bresnick *et al.*, 1991).

The two EF hand motifs of Dd plastin are more closely related to the Ca^{2+}-binding sites of calmodulins from various organisms than to members of the fimbrin/plastin family from other species. In the helix-loop-helix motifs of both EF hands, the amino acid residues involved in the coordination of Ca^{2+} (Kretsinger, 1980) are conserved in Dd plastin.

Specificity of the Binding of Dd Plastin to F-Actin and Its Inhibition by Ca^{2+}

Dd plastin turned out to bind to filaments of β/γ-actin from bovine spleen, but not of α-actin purified from rabbit skeletal muscle (Figure 3A). Not by high-speed centrifugation, light-scattering measurements, nor viscometry was any significant interaction of Dd plastin with the muscle actin observed under conditions where unambiguous binding to *D. discoideum* actin was found. The absence or presence of Ca^{2+} had no influence on these negative results.

In accord with the presence of two perfect EF hands, the binding of Dd plastin to actin from *D. discoideum* proved to be sensitive to Ca^{2+}, with an inhibition by 92% at 200 μM Ca^{2+} (Figure 3B). Mg^{2+} up to a concentration of 5 mM did not inhibit binding of Dd plastin to the F-actin, and 2 mM Mg^{2+} did not prevent the inhibition of binding caused by 200 μM Ca^{2+}.

The amount of Dd plastin bound to F-actin in the absence of Ca^{2+} decreased with increasing KCl concentrations. At 100 mM KCl, a concentration routinely used for actin polymerization, binding was reduced by 43%. The slightly chaotropic Cl^- ions are kept at low concentration within cells (Richey *et al.*, 1987). When Cl^- was substituted by glutamate to approximate physiological conditions (de Hostos *et al.*, 1991), no inhibition of the binding up to 100 mM potassium glutamate was observed; at 75 mM potassium glutamate the binding was even enhanced.

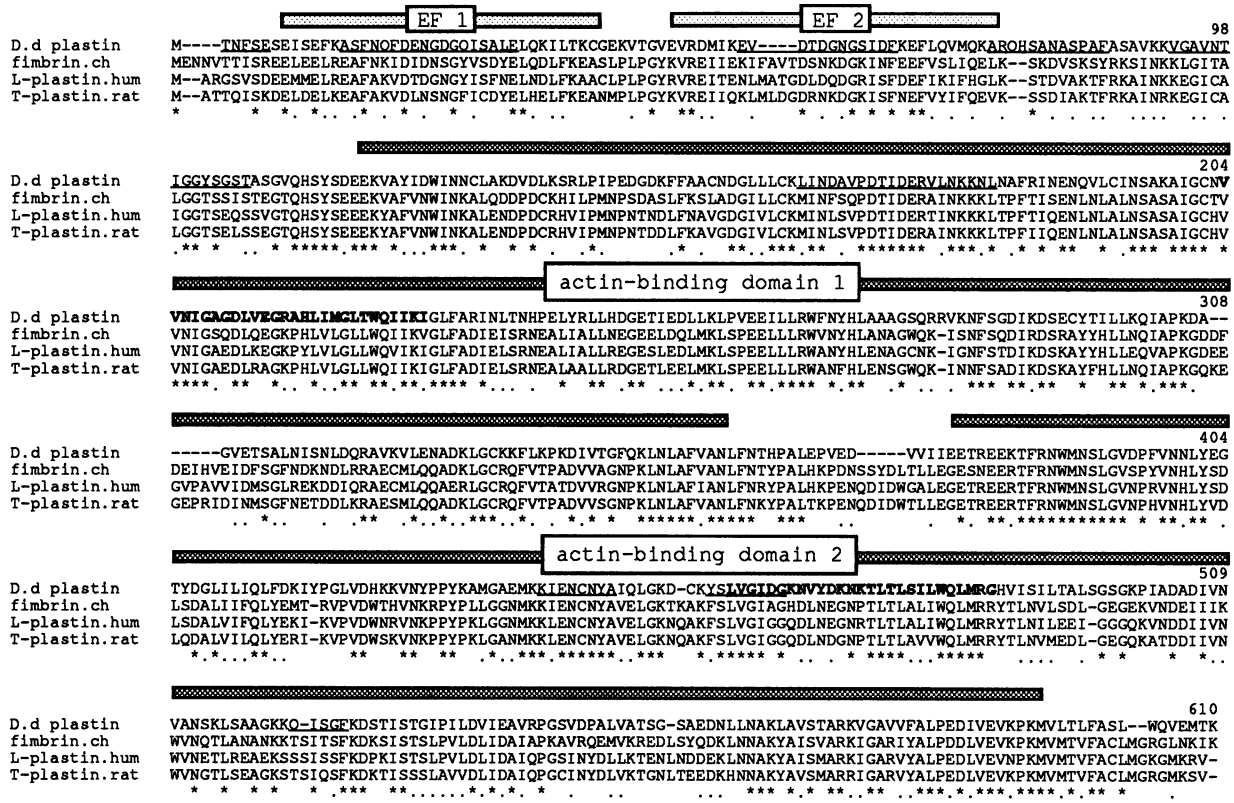

Figure 2. Comparison of the cDNA-derived amino acid sequence of Dd plastin (p67) with vertebrate members of the plastin/fimbrin family. Identical amino acids are marked by asterisks, conserved residues by dots. The domain structure of Dd plastin is indicated by boxes on top of its sequence. A conserved stretch of 27 amino acids in each of the two actin-binding domains is indicated by bold letters. Underlined portions of the sequence were confirmed by direct amino acid sequencing of peptides obtained by Lys-C cleavage of Dd plastin. Sequences of vertebrate plastins/fimbrins were obtained from the Protein Identification Resource Data Bank under accession numbers A37097 (chicken brush border fimbrin), A35836 (human L-plastin), and S31765 (rat T-plastin). Dd plastin has received the GenBank accession number L36202.

An equilibrium binding constant of Dd plastin with F-actin was determined by high-speed centrifugation in the presence of EGTA (Figure 3C). Cosedimentation data were fitted using a Scatchard plot with two straight lines, one indicating a K_d of 5×10^{-7} M and a maximal ratio of 1 Dd plastin monomer bound per 5 protomers of actin. The other line suggests that Dd plastin can bind with lower affinity to actin at a ratio of one plastin molecule to 2.5 actin protomers. Different affinities of the two actin-binding domains in a plastin molecule might be responsible for nonlinearity of the binding curve. Low-affinity binding may, however, also result from the involvement of only a single binding site of the Dd plastin molecule at high plastin to actin ratios.

Cross-Linking of Actin Filaments by Dd Plastin

Evidence that Dd plastin efficiently cross-links actin filaments in a Ca^{2+}-inhibited manner was provided by cosedimentation, light scattering, and viscometry assays. Light scattering in a solution of F-actin was strongly increased by Dd plastin, with a maximal effect at a plastin monomer to actin protomer ratio of 1:5 (Figure 4A). The increase in light scattering was immediately reversed by the addition of Ca^{2+} and was recovered by adding EGTA (Figure 4B), thus excluding the possibility that the average length of actin filaments was altered.

After incubation of 6.0 μM actin with 1.5 μM Dd plastin under polymerizing conditions in the presence of EGTA, >80% of the actin was pelleted at low *g* force, whereas only a negligible fraction of actin filaments was pelleted without plastin (Figure 4C). By the falling-ball method, a marked increase in viscosity was found at a monomer to protomer ratio of 1:16, and at a 1:5 ratio the gel became too rigid for the ball to move (Figure 4D). The Dd plastin to actin ratio was increased up to equimolarity, and the viscosity remained unmeasurably high under these conditions.

In Vitro Motility Assay Showing Actin Filament Bundles to be Longitudinally Cleaved by the Motor Action of Myosin

Complexes of Dd plastin with TRITC-phalloidin-labeled actin filaments were immobilized on glass surfaces coated with heavy meromyosin under conditions where the actin alone formed single filaments (Figure 5A). At a Dd plastin to actin ratio of 1:20, fluorescent bundles but no networks of cross-linked filaments were seen (Figure 5B). The bundles were distinguishable from single actin filaments by more intense fluorescence emission, most clearly when the actin filaments were attached to each other only at portions of their entire length. With increasing the Dd plastin to actin ratio up to 1:2, the bundles became thicker and often large clusters of actin filaments were formed which displayed no obvious regularity in their organization (Figure 5C).

No obvious disassembly of actin bundles and clusters was observed within 20 min when the solution in the chamber was replaced by fresh, actin-free, and plastin-free buffer. The stability of actin-plastin complexes was however broken within seconds upon the release of ATP from caged ATP due to force generation of the heavy meromyosin motor.

Single filaments were propelled out of a bundle (Figure 6A), and even large actin clusters were disaggregated within 30 s into single sliding filaments (Figure 6B).

Distribution of Dd Plastin in Cells of Different Developmental Stages

Growing cells of *D. discoideum* are irregular in shape, and they often change the direction of pseudopod protrusion. Aggregating cells tend to become elongated, and they are characterized by an expanding leading edge at one end and a contracting tail at the other. The localization of Dd plastin in cells of the two stages of development was determined using immunolabeling with monoclonal or affinity-purified polyclonal antibodies specific for Dd plastin, as shown in Figure 1C.

For immunolabeling, the cells were fixed either with methanol at −20°C, which only slightly denatures proteins but poorly preserves cell shape and intracellular structures, or with picric acid/formaldehyde, which is a stronger fixative particularly suited for *Dictyostelium* cells (Brink *et al.*, 1990). Differences in the labeling pattern due to the choice of the fixative were most obvious in growth phase cells. After fixation with picric acid/formaldehyde, crown-shaped extensions on the dorsal surface and the leading edges of the cells were most intensely labeled (Figure 7). Cells fixed in methanol were distinguished by the strong labeling of filopods along their entire length (Figure 8, A and B). In contrast, cells fixed with picric acid/formaldehyde showed no prominent labeling of filopods, except sometimes at their base (Figure 8, C and D).

In aggregating cells, Dd plastin was found to be enriched at the leading edges after treatment with either one of the fixatives (Figure 8, E–H). Dd plastin was found to be distributed throughout the cytoplasm at both stages of cell development, but the label in the cytoplasmic space was much weaker after methanol fixation than in cells fixed with picric acid/formaldehyde (Figure 8, B and F compared with D and H).

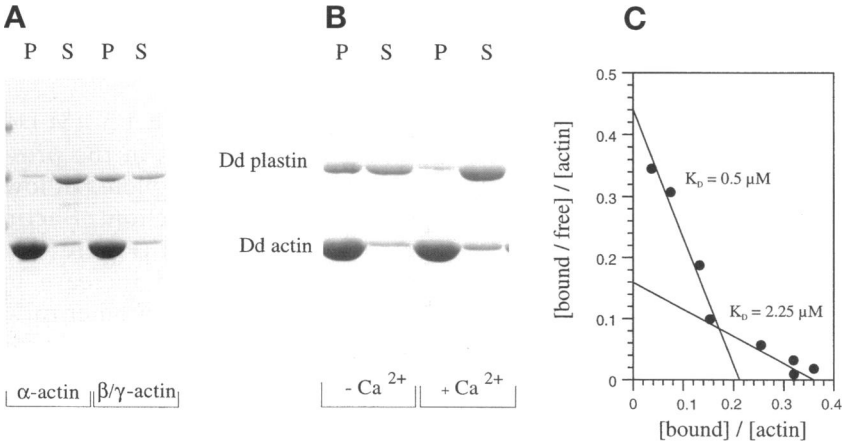

Figure 3. Binding of Dd plastin to F-actin. (A) Cosedimentation assay of Dd plastin with rabbit skeletal muscle actin (α-actin) and with bovine spleen actin (β/γ-actin). Actin (6 μM) was incubated with 1 μM Dd plastin in polymerization buffer containing 2 mM $MgCl_2$ and 1 mM EGTA. (B) Effect of Ca^{2+} on Dd plastin binding to *D. discoideum* actin. Actin (6 μM) from *D. discoideum* was incubated with 2.5 μM Dd plastin in polymerization buffer containing 2 mM $MgCl_2$ and either 1 mM EGTA ($-Ca^{2+}$) or 200 μM $CaCl_2$ ($+Ca^{2+}$). (C) Scatchard plot of binding data obtained in the absence of Ca^{2+} under the same conditions as in A. Pellets (P) and supernatants (S) were separated by high-speed centrifugation, and the proteins in these fractions were analyzed by SDS-PAGE in 12% gels and staining with Coomassie blue. For C, the stained gels were scanned and concentrations calculated in μM of Dd plastin or actin. Binding was assayed in the range of 0.75–24 μM Dd plastin to 6 μM actin.

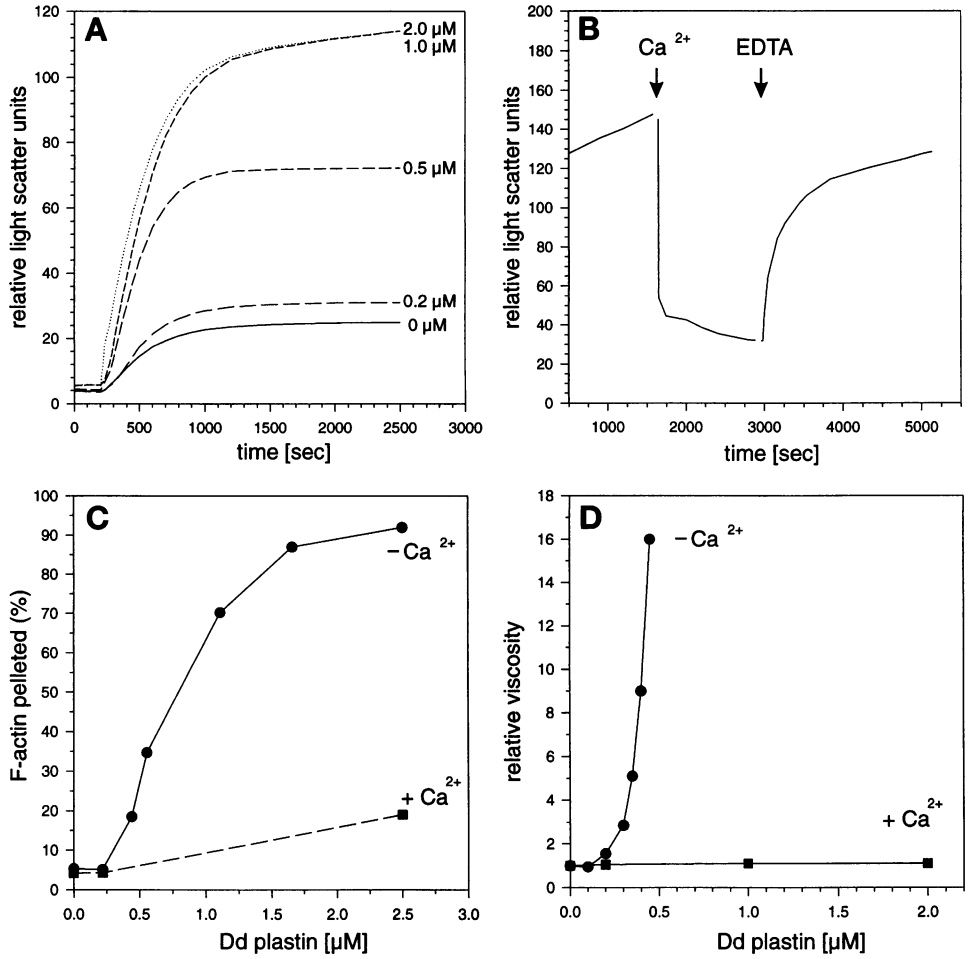

Figure 4. F-actin cross-linking activity of Dd plastin and its inhibition by Ca^{2+}. (A) Effect of various concentrations of Dd plastin on light scattering during actin polymerization in the presence of EGTA. (B) Kinetics of reversible changes in light scattering induced by the addition of 2 mM $CaCl_2$ and subsequent addition of 5 mM EGTA. Actin had been prepolymerized in the presence of 1 µM Dd plastin and 1 mM EGTA. Recovery of light scattering after the addition of EGTA, which indicates reformation of cross-links between the polymerized actin, occurred with a half-time of about 4 min. In A and B, the concentration of actin from *D. discoideum* was 5 µM. Zero on the abscissa is the time when actin polymerization was started. (C) Low-speed sedimentation of actin as a function of the Dd plastin concentration. The actin had been preincubated for 2 h at room temperature with Dd plastin in polymerization buffer containing either 1 mM EGTA ($-Ca^{2+}$) or 200 µM $CaCl_2$ ($+Ca^{2+}$). The actin concentration was 6 µM; actin in pellets and supernatants was quantified as in Figure 3C. (D) Low-shear viscometry of polymerized actin as a function of the Dd plastin concentration in the presence and absence of Ca^{2+}. The actin concentration was 5 µM, and the buffer conditions were as in C.

DISCUSSION

Another Ca^{2+}-regulated Actin-Bundling Protein in Dictyostelium

In vertebrates, isoforms of plastins/fimbrins are expressed in a cell-type specific manner, and different roles in the organization of the actin skeleton have been attributed to these isoforms (Arpin *et al.*, 1994). I-plastin is expressed in the intestine and kidney (Lin *et al.*, 1994). L-plastin is restricted to rapidly moving cells, such as lymphocytes and macrophages, and to neoplastic cells derived from solid tissue, in which T-plastin is normally found (Goldstein *et al.*, 1985; Lin *et al.*, 1988). The switch from T-plastin to L-plastin is paralleled by morphological changes from flat to more rounded cells and by alterations in the organization of the microfilament system.

Along with a 30-kDa F-actin bundling protein (Fechheimer and Taylor, 1984) and α-actinin (Noegel *et al.*, 1989), Dd plastin is one of three actin-cross-linking proteins known from *D. discoideum* cells that are inhibited by Ca^{2+}. This parallel regulation may be interpreted to mean that, by multiple controls, actin cross-linkages are disrupted in response to signals that cause an increase in cytoplasmic Ca^{2+}. In the large eosinophils of newts, a transient and local increase of cytoplasmic Ca^{2+} has been found at the rear of chemotactically stimulated cells (Brundage *et al.*, 1993). In the small cells of *Dictyostelium*, such a local increase has not been detected, but a chemoattractant-induced Ca^{2+} influx and release of Ca^{2+} from intracellular vesicles indicates a transient rise of cytoplasmic Ca^{2+} within 5 s of stimulation (Saran *et al.*, 1994). At the beginning of a chemotactic response, blebbing of the cell surface accompanied by a slight contraction of the cell body is often observed in video recordings (Gerisch, unpublished results). The formation of rounded blebs instead of structured pseudopods indicates a local decrease in the rigidity of the cell cortex. This response may be based on the concerted inactivation of F-actin cross-linking proteins by Ca^{2+} at the initiation of a chemotactic response.

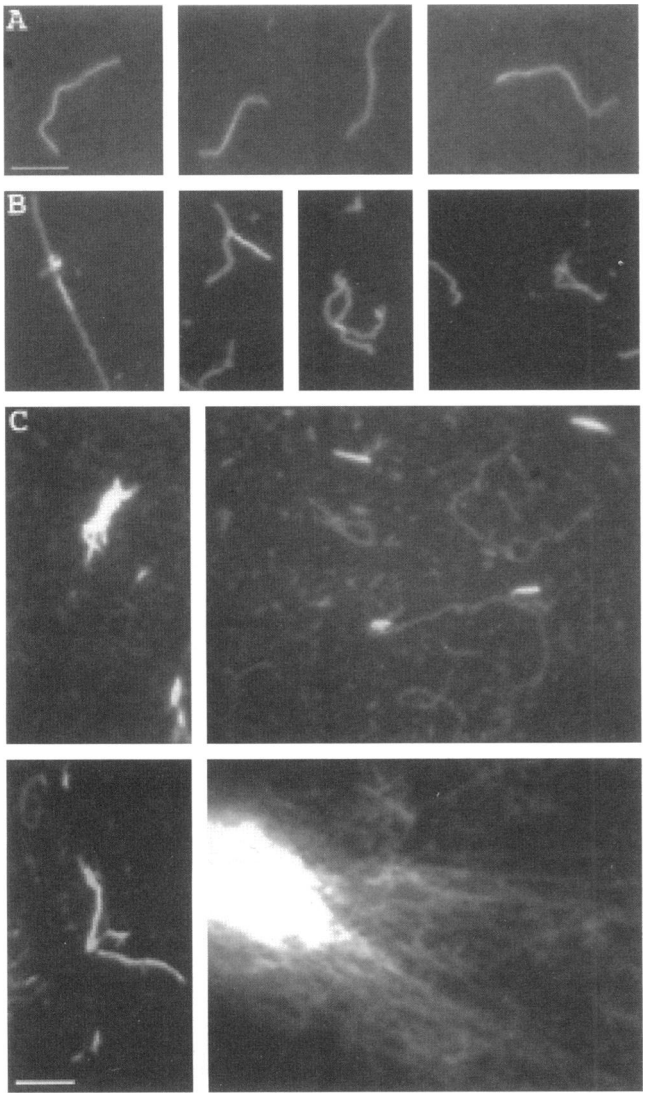

Figure 5. Complexes of actin filaments with Dd plastin visualized by fluorescence microscopy. Actin (10 μM) from *D. discoideum* was polymerized, labeled with TRITC-phalloidin, and incubated without Dd plastin (A) or with 0.5 μM (B) or 5 μM Dd plastin (C). Before microscopy, the actin was immobilized on heavy meromyosin-coated glass. A shows single actin filaments; B, filaments bundled into dimers along part of their length. The four panels of C exemplify bundles of more than two actin filaments and larger, less ordered complexes. Bars, 10 μm.

Specificity of Binding to Actin

Members of the plastin/fimbrin family differ in the specificity of their interactions with isoforms of actin. I-plastin binds to α-actin from rabbit muscle (Lin *et al.*, 1994), and T-plastin binds not only to α-actin but also to β- actin and γ-actin (Arpin *et al.*, 1994). Conversely, L-plastin binds to β-actin from human T-cells, but not to rabbit α-actin (Namba *et al.*, 1992). The Sac6 protein from yeast was isolated by affinity chromatography on a yeast F-actin column but could not be purified on a rabbit α-actin column (Drubin *et al.*, 1988).

The rapidly moving cells of *D. discoideum* most closely resemble, in the dynamic rearrangements of their actin skeleton, the L-plastin-producing cells of the hematopoietic system. The binding to β/γ-actin, in combination with the lack of binding to α-actin from rabbit skeletal muscle, relates the Dd plastin to L-plastin and distinguishes it from the T- and I-plastins. Therefore, we consider Dd plastin to be closely related in its specificity to the L-plastin of hematopoietic cells.

Profilin and myosin II of *D. discoideum* show different affinities for actin from various sources (Greer and Schekman, 1982; Haugwitz *et al.*, 1991), but Dd plastin is the first protein known from this organism that does not bind actin from rabbit skeletal muscle to any significant extent. The protein appears therefore to bind to a region where the major actin (actin 15) of *D. discoideum* (Vandekerckhove and Weber, 1980) differs in its structure from rabbit α-actin. Three variable regions become obvious when the sequences of *D. discoideum* actin 15 or human β-actin are compared with rabbit α-actin. One region is located in the central β-sheet of subdomain 3, another at the bridge of subdomains 3 and 4, and a third at the lower right-hand side of subdomain 1 (Hennessey *et al.*, 1993). Based on the mutagenesis of yeast actin, a binding site for yeast Sac6p has been proposed to reside in subdomain 1 near subdomain 2 (Holtzman *et al.*, 1994; Honts *et al.*, 1994). In the atomic structure of the actin molecule (Kabsch *et al.*, 1990), this proposed binding site is situated in close vicinity to the cluster of variable amino acid residues in subdomain 1.

Association of Members of the Plastin/Fimbrin Family with Cellular Structures

All members of the fimbrin/plastin family are soluble cytoplasmic proteins that bind to actin-rich intracellular structures. Chicken intestinal fimbrin was one of the first actin-binding proteins found to be primarily associated with microvilli and other cell protrusions rather than with the stress fiber system (Bretscher and Weber, 1980). Labeling of T-plastin in the fibroblast-like cell line CV-1 showed, in addition to diffuse accumulation in the actin-rich cell cortex, a strong enrichment of the protein in filopods and at focal contacts (Arpin *et al.*, 1994).

The immunolabeling pattern of Dd plastin proved to depend on the fixative used. One reason appears to be that after fixation with cold methanol, soluble proteins like Dd plastin are partially extracted from the fixed cells and might even redistribute to structures to which they are not bound within the living cell. Stronger fixation with a picric acid/formaldehyde mixture, on the other hand, may prevent antibodies from getting access to their binding sites within complexes of

tightly connected proteins. Because of these ambiguities, we consider only structures strongly labeled after both fixations as being reliably enriched in Dd plastin. Structures of primary interest are the crowns on the top surface and the lamellipods of growth phase cells and the leading edges of aggregating cells. At these transient extensions of the cell surface, Dd plastin is recruited from its cytoplasmic pool and integrated into the network of actin filaments. Accumulation at the cell-to-substrate contact area, as found for T-plastin or L-plastin in fibroblasts or macrophages, was not observed in confocal images of *D. discoideum* cells (Figure 7).

Extrapolation of the Data on Actin Bundling and Cross-Linkage to Conditions In Vivo

By comparing immunoblots from cell homogenates and from purified Dd plastin as a standard, a rough estimate of 0.6 μM was obtained for the concentration of the bundling protein in the cytoplasm. At this concentration Dd plastin strongly increased the viscosity of F-actin in vitro (Figure 4D). Our data suggest that about one half of the Dd plastin is soluble and one half is actin bound in living cells. This notion is based on a K_d in the range of 5×10^{-7} M (Figure 3C), neglecting the presence of other proteins that might compete for binding sites on the actin and assuming an actin concentration of 1×10^{-4} M in the cell (calculated as monomer), one half of that being polymerized (Hug *et al.*, 1995). The data are in accord with the results of immunofluorescence labeling that showed part of Dd plastin to be in the cytoplasmic space and a substantial portion to be concentrated in cortical, actin-rich structures. Thus, Dd plastin is present in the cells at a concentration appropriate for its actin cross-linking activity to be efficiently regulated by Ca^{2+}. Gene disruption experiments are underway to examine the contribution of Dd plastin to cell behavior in cells with or without defects in other actin cross-linking proteins.

The high viscosity of actin, even at a low concentration of Dd plastin, indicates that the cross-linking is not simply bundling. In fact, we have found from electron microscopic analysis that one or more filaments from one bundle are connected and integrated into other bundles, resulting in an extensive network of bundled actin filaments (our unpublished data). This is similar to the network depicted for cortexillin I, another actin filament bundling protein from *D. discoideum* (Faix *et al.*, 1996). Increasing the ratio of Dd plastin to actin did not result in a collapse of the actin filament network, in variance to what has been reported for the 30-kDa actin bundling protein by Fechheimer (1987). Even at a Dd plastin to actin ratio of 1:1, viscosity remained high, which indicates that with a ratio much higher than those in living cells the Dd plastin still bundles actin filaments with a geometry that allows the formation of a network of actin filaments.

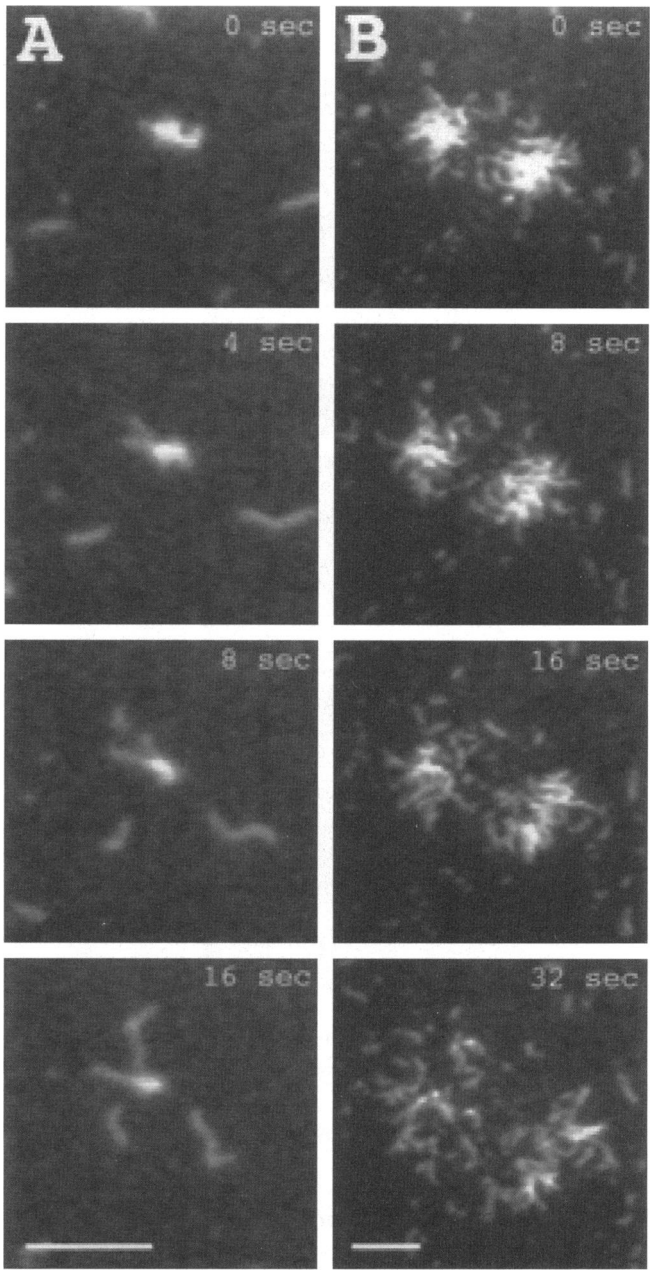

Figure 6. In vitro motility assay using complexes of actin with Dd plastin. TRITC-phalloidin-labeled filaments of *D. discoideum* actin incubated with 0.5 μM (A) or 5 μM (B) Dd plastin were bound to heavy meromyosin-coated glass as in Figure 5 and washed with buffer containing 1 mM caged ATP. At zero time, ATP was released by an UV flash, and images were taken from video recordings at the times indicated. In A, an actin filament bundle cleaved by the myosin heads after ATP release into single filaments is seen to move toward the left hand side. In B, two larger aggregates of actin filaments are dispersed by the ATP-activated myosin heads.

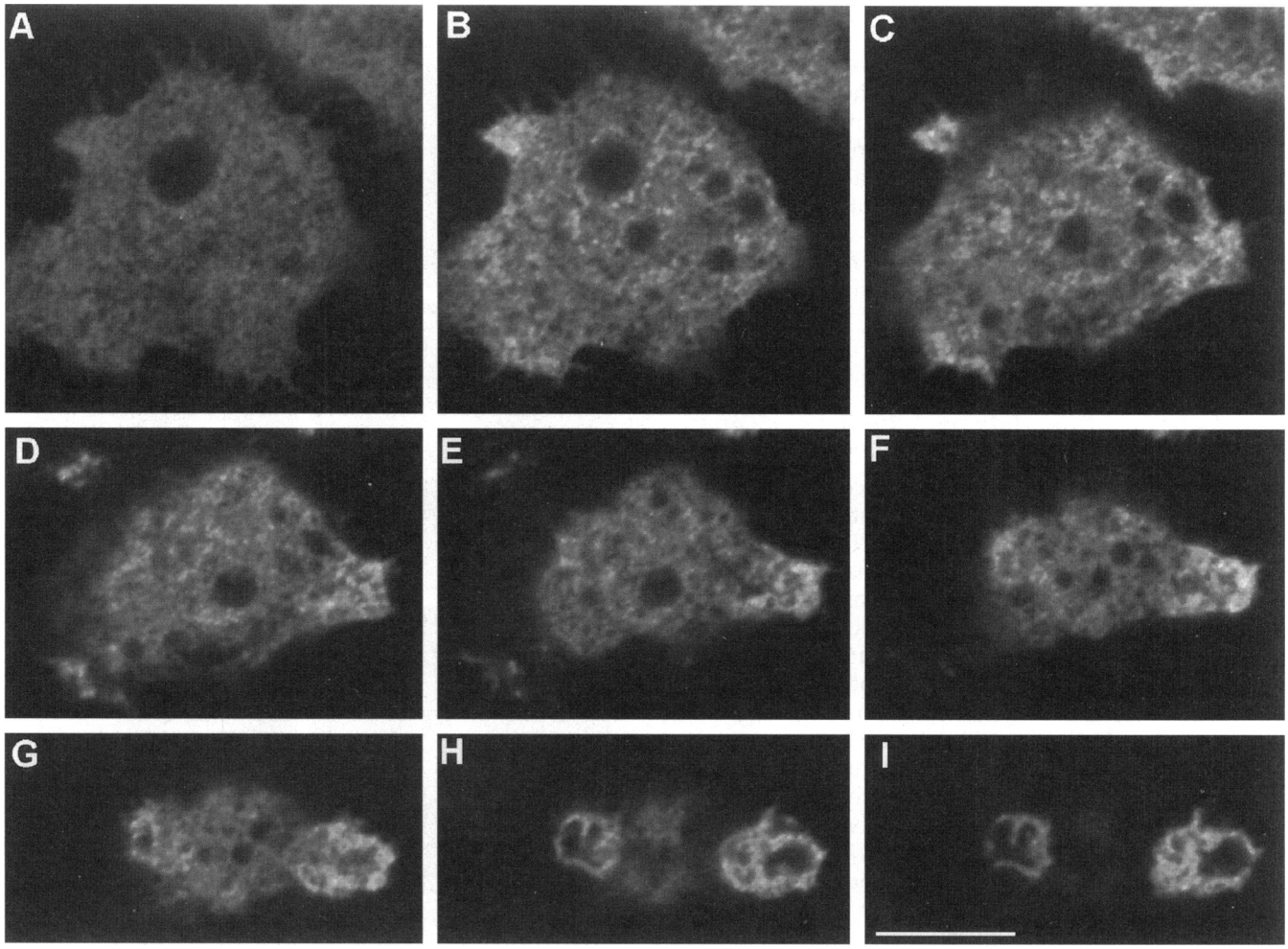

Figure 7. Series of confocal fluorescence images through a growth phase cell of *D. discoideum* labeled with anti-Dd plastin antibody. The cell attached to glass was fixed using the picric acid/formaldehyde procedure. Dd plastin was indirectly labeled with mAb 210–183-1 and TRITC-conjugated goat anti-mouse IgG. Confocal sections were taken at intervals of 0.6 μm from the basal cell surface on glass (A) to the free upper surface (I) where two crown-shaped extensions are pointing toward the fluid space. Extensions of the cell, including crowns, are more strongly labeled than the cytoplasm, but no accumulation of Dd plastin at the basal surface is recognizable. Bar, 10 μm.

Dissociation of Actin Bundles by Myosin

In living cells, Dd plastin acts in concert with numerous other proteins that cause actin filaments to assemble into networks or bundles. These cross-bridging proteins stabilize the actin cortex in a way that does not prevent the cells from rapidly changing their shape during migration and food uptake. A major question in this context is whether myosins are capable of moving along actin filaments that are laterally connected to each other. Our results indicate that myosin II can act as a motor using actin filament bundles as a substrate, but the myosin does not translocate the bundles as a whole. The force exerted by the myosin heads on the bundles disrupts them from the minus toward the plus end into single filaments which then move independently of each other.

For the in vitro motility measurements, a solution of 10 μM F-actin and 6 μM Dd plastin was diluted 50 times into the chamber. If we were to assume no substantial dissociation occurred after the dilution, then based on a K_d of 5×10^{-7} M we would expect, as an upper estimate, an almost saturating number of cross-bridges: 1 Dd plastin to 5 actin protomers or 80 molecules of Dd plastin per μm of two cross-linked actin filaments (2×200 protomers). Although the protein concentration in the chamber is below the equilibrium constant, dissociation of Dd plastin may be retarded because of the close proximity of the filaments, such that a dissociation event will be followed by rebinding at the same site. This might explain our observation that massive complexes of cross-linked filaments can be seen even 20 min after immobiliza-

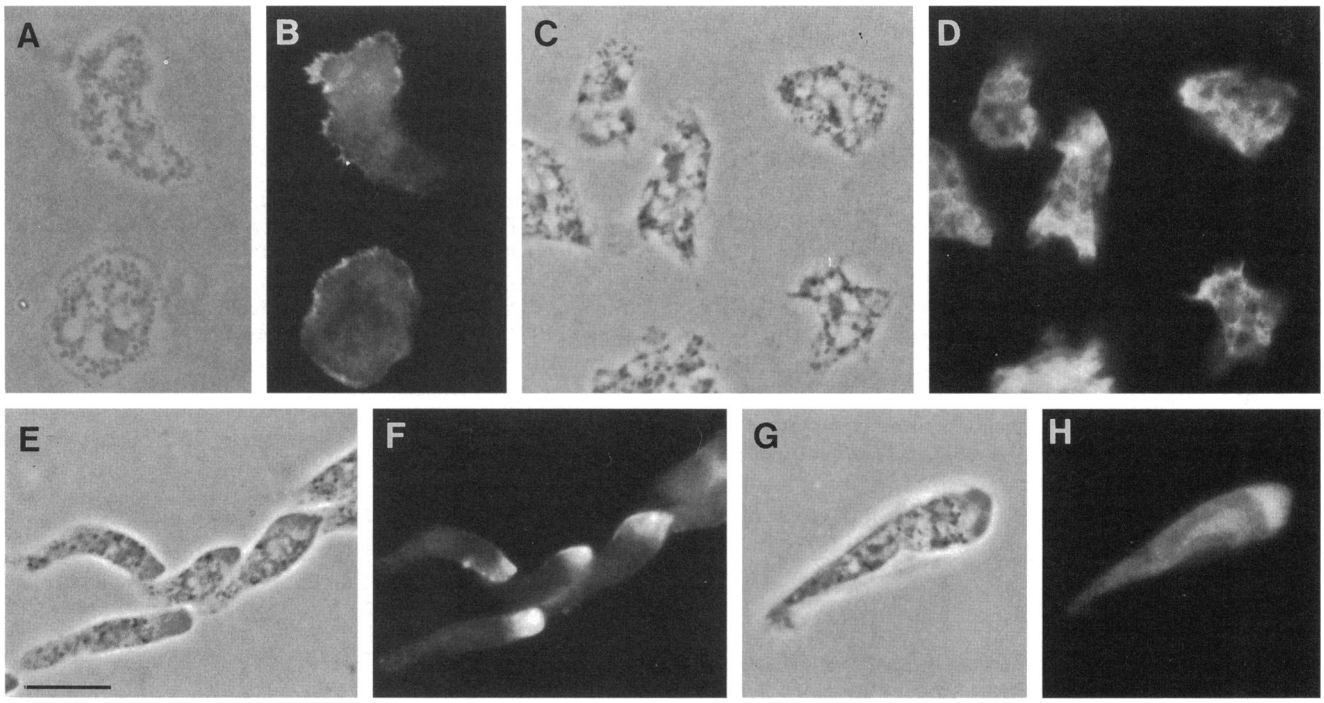

Figure 8. Immunofluorescence localization of Dd plastin in growth phase cells (A–D) and in aggregation-competent cells (E–F) of *D. discoideum*. For A, B, and E, F cells were fixed with cold methanol, for C, D, and G, H with picric acid/formaldehyde followed by 70% ethanol. Cells were labeled as in Figure 7 and corresponding phase-contrast micrographs (A, C, E, and G) and fluorescence images (B, D, F, and H) are shown. In A and E, the extraction of soluble proteins from the cytoplasm is evident when these micrographs are compared with those of C and G. Bar, 10 μm.

tion on the HMM surface in the absence of ATP. Given a K_d of the interaction between Dd plastin and actin of 5×10^{-7} M, a free energy of binding of 35 KJ/mol is derived. In a living cell the hydrolysis of ATP proceeds with a free energy of 52 KJ/mol. It therefore appears that the energy available from the hydrolysis of a single ATP by the actomyosin ATPase is sufficient to break a Dd plastin-actin bond. However, multiple power strokes may be necessary to overcome the effect of rebinding and of the load on the actin filaments induced by the 80 or less cross-links per μm. It might also be considered that once the front ends of two cross-linked filaments are free and drawn away from each other, the myosin heads working on these ends are more effective at diminishing the load on the system by successively unzipping the remaining cross-links at the back end.

The separation of actin filaments out of the bundle shows that myosin has access to actin filaments that are cross-linked by Dd plastin and is capable of dispersing clusters of cross-linked actin filaments. It will be of interest to find out using the in vitro motility system how myosin handles actin networks that are made of other proteins and to analyze the interaction of myosin with complexes of actin filaments that are produced by more than a single species of cross-linking proteins.

The actin-myosin system in the cortex of a motile cell has the dual function of stabilizing the cell's shape and to alter that shape. Theoretical and experimental studies in vitro have addressed the question of how these functions are regulated and coordinated in vivo by proteins that are responsible for controlling the polymerization of actin and the cross-linkage of its filaments. Two assumptions, which are not necessarily mutually exclusive, are 1) that contraction requires partial dissociation of cross-bridges between actin filaments [solation-contraction coupling (Hellewell and Taylor, 1979)] and 2) that cross-linkages between actin filaments amplify the contraction brought about by myosin [rigidity-shear hypothesis (Stossel, 1982)]. In both cases, the state of cross-linkage of the actin filaments is supposed to be independent of the motor activity of myosin.

In the present study, F-actin bundles and networks formed by Dd plastin are shown to be dissociated into single filaments by the ATP-dependent motor activity of myosin. Our results suggest that solation is coupled to myosin-mediated contraction, an effect that mirrors the classical coupling of contraction to solation

(Hellewell and Taylor, 1979). Our results differ from those of a previous study (Janson *et al.*, 1992) in which two F-actin cross-linking proteins, nonmuscle α-actinin and filamin, were bound, along with the myosin heads, to a solid support. Under these conditions, the actin filaments added were primarily arrested at the surface rather than cross-linked to each other. As a consequence, myosin-driven movement of these filaments was strongly inhibited even at low cross-linker to myosin ratios. In our experiments, the Dd plastin was applied to the actin filaments in solution, which is similar to how Dd plastin is recruited within the living cell from the cytosol to the cortical system of actin filaments. The results obtained under these conditions indicate that motor proteins can alter the state of the actin network on which they act. It might be important to consider this possibility in deducing the behavior of the cortical actin system from the activities of the proteins that control its function in a motile cell.

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