Dictyostelium discoideum Cells Lacking the 34,000-Dalton Actin-binding Protein Can Grow, Locomote, and Develop, but Exhibit Defects in Regulation of Cell Structure and Movement: A Case of Partial Redundancy

F. Rivero,* R. Furukawa,‡ A.A. Noegel,* and M. Fechheimer‡

*Max-Planck-Institute for Biochemistry, 82152 Martinsried, Germany; and ‡Department of Cellular Biology, University of Georgia, Athens, Georgia 30602

Abstract. Cells lacking the *Dictyostelium* 34,000-D actin-bundling protein, a calcium-regulated actin cross-linking protein, were created to probe the function of this polypeptide in living cells. Gene replacement vectors were constructed by inserting either the UMP synthase or hygromycin resistance cassette into cloned 4-kb genomic DNA containing sequences encoding the 34-kD protein. After transformation and growth under appropriate selection, cells lacking the protein were analyzed by PCR analyses on genomic DNA, Northern blotting, and Western blotting. Cells lacking the 34-kD protein were obtained in strains derived from AX2 and AX3. Growth, pinocytosis, morphogenesis, and expression of developmentally regulated genes is normal in cells lacking the 34-kD protein. In chemotaxis studies, 34-kD$^-$ cells were able to locomote and orient normally, but showed an increased persistence of motility. The 34-kD$^-$ cells also lost bits of cytoplasm during locomotion. The 34-kD$^-$ cells exhibited either an excessive number of long and branched filopodia, or a decrease in filopodial length and an increase in the total number of filopodia per cell depending on the strain. Reexpression of the 34-kD protein in the AX2-derived strain led to a "rescue" of the defect in the persistence of motility and of the excess numbers of long and branched filopodia, demonstrating that these defects result from the absence of the 34-kD protein. We explain the results through a model of partial functional redundancy. Numerous other actin cross-linking proteins in *Dictyostelium* may be able to substitute for some functions of the 34-kD protein in the 34-kD$^-$ cells. The observed phenotype is presumed to result from functions that cannot be adequately supplanted by a substitution of another actin cross-linking protein. We conclude that the 34-kD actin-bundling protein is not essential for growth, but plays an important role in dynamic control of cell shape and cytoplasmic structure.

COORDINATION of cell movements requires spatial and temporal control of the structure and consistency of the cytoplasm, as well as the distribution and activity of a variety of myosins. Changes in cytoplasmic consistency producing reversible gel to sol transitions appear to be mediated largely by control of the interactions of actin and a variety of actin cross-linking proteins (Taylor and Condeelis, 1979; Taylor and Fechheimer, 1982). Characterization of actin cross-linking proteins has revealed a number of families of proteins whose structure and activity has been highly conserved among the eukaryotes (Matsudaira, 1991; Otto, 1994). Characterization of the structure and functions of these actin cross-linking proteins is essential to an understanding of the control of changes in cytoplasmic structure during cell movements.

The cellular slime mold *Dictyostelium discoideum* has emerged as an organism that is singularly well suited to the study of the structure and function of cytoskeletal proteins because of the unique combination of cell biological, biochemical, and molecular genetic approaches that can be used in this system (Mann et al., 1994; Schleicher and Noegel, 1992). Eight different types of actin cross-linking proteins have been identified in *Dictyostelium*, including a filamin-like protein (Hock and Condeelis, 1987), spectrin-like protein (Bennett and Condeelis, 1988), a 120-kD protein termed gelation factor (Condeelis et al., 1981; Noegel et al., 1989), α-actinin (Fechheimer et al., 1982; Condeelis and Vahey, 1982; Noegel et al., 1987), elongation factor 1α (Demma et al., 1990), comitin (Weiner et al., 1993), and two low molecular weight actin-bundling proteins with apparent molecular masses of 30 kD (Brown, 1985) and 34 kD

Please address all correspondence to Dr. Marcus Fechheimer, Department of Cellular Biology, University of Georgia, Athens, GA 30602. Tel.: (706) 542-3338; Fax: (706) 542-4271; E-mail: Fechheimer@cb.uga.edu

© The Rockefeller University Press, 0021-9525/96/11/965/16 $2.00
The Journal of Cell Biology, Volume 135, Number 4, November 1996 965–980

(Fechheimer, 1993), respectively. The ability to test the contribution of single proteins to cell movements has been used to produce cell lines that lack either α-actinin or the gelation factor, resulting in cells with no defects or moderate perturbations of the ability to move, grow, and complete the developmental cycle (Witke et al., 1987; Brink et al., 1990). Recent investigations reveal that cells lacking the 120-kD protein derived from *Dictyostelium* AX3 show more pronounced defects than those obtained previously from AX2 (Cox et al., 1992, 1995, 1996).

The *Dictyostelium* 34-kD actin-bundling protein cross-links actin filaments into bundles in vitro in the presence of low but not micromolar concentrations of free calcium ion (Fechheimer and Taylor, 1984; Fechheimer, 1987). The sequence of the protein deduced from its cDNA contains 295 amino acids with two putative EF hands that may mediate calcium binding (Fechheimer et al., 1991). This protein has been found to inhibit the disassembly of actin filaments, suggesting that it may selectively stabilize actin filaments in networks with which it is associated (Zigmond et al., 1992). It has been localized in the filopodia and pseudopodia, phagocytic cup, cleavage furrow, and cell–cell contact sites, suggesting participation in diverse cell movements during the *Dictyostelium* life cycle (Fechheimer, 1987; Fechheimer et al., 1994; Furukawa et al., 1992; Furukawa and Fechheimer, 1994; Johns et al., 1988; Okazaki and Yumura, 1995). Homologues of the 34-kD protein have been described in *Physarum* and in mammalian cells (St.-Pierre et al., 1993; Johns et al., 1988).

We have created *Dictyostelium* cell lines lacking the 34-kD protein by gene replacement to test its potential contributions to cell structure and movement. The results reveal that the cells lacking the 34-kD protein can grow and develop normally, show increased persistence of motility, shed bits of cytosol while migrating on a solid substrate, and exhibit either abnormally long and branched filopodia or increased numbers of filopodia, depending on the strain of *Dictyostelium*. While the conserved structure and specific localization of the 34-kD protein indicates that it plays a distinct role in cell structure and movement, the concept of functional redundancy cannot be excluded in explaining the initial results from the gene replacement experiments.

Materials and Methods

Dictyostelium Strains and Growth Conditions

Cells of strain AX2-214 (referred to as wild type) and mutant strains were grown either in liquid nutrient medium at 21°C with shaking at 160 rpm (Claviez et al., 1982) or on SM agar plates with *Klebsiella aerogenes* (Williams and Newell, 1976). Strain DH1 (derived from AX3; Caterina et al., 1994), containing a deletion in the *Dictyostelium* pyr 5-6 locus encoding UMP synthase, was grown in synthetic medium FM (Franke and Kessin, 1977), which was purchased from Sigma Chemical Co. (St. Louis, MO) and supplemented with 20 μg/ml uracil.

Preparation of the Gene Replacement Vectors

A 4-kb genomic DNA containing sequences encoding the *Dictyostelium* 34,000-D actin-bundling protein was isolated for construction of the gene replacement vectors. Genomic DNA of strain AX3 was isolated from the nuclei and banded in cesium chloride/ethidium bromide gradients, as described (Noegel et al., 1985). The DNA was digested with a mixture of BamHI and BglII, resolved in an agarose gel, transferred to nitrocellulose, and probed with the cloned cDNA (Fechheimer et al., 1991) labeled by the method of random primers (Feinberg and Vogelstein, 1983) to identify the size of the genomic DNA of interest. Specific conditions used for hybridization and washing of the blots were as described previously (Fechheimer et al., 1991). The region around 4 kb containing the sequences of interest was excised, ligated into pBluescriptSK$^-$ that had been previously digested with BamHI, treated with calf alkaline phosphatase, and used to transform SURE cells. Colony blots were probed with the radiolabeled cDNA sequences described above. The clones were mapped and sequenced in the region around the coding region, revealing a short intron slightly downstream from the translational start codon (Fig. 1). These sequence data are available under GenBank accession number U32112 and EMBL sequence database accession number Z50156.

The gene replacement vector containing the hygromycin resistance marker was prepared by first digesting the genomic DNA at the unique NsiI site within the coding region and blunting the ends of the linearized DNA using T4 DNA polymerase. The 1.8-kb hygromycin resistance cassette, retrieved initially from pDe109 (Egelhoff et al., 1989), was blunt ended using T4 DNA polymerase, ligated with the genomic DNA prepared as described above, and used to transform SURE cells. This gene replacement vector is shown in Fig. 2.

The gene replacement vector containing the *Dictyostelium* pyr 5-6 (UMP synthase) gene was prepared as follows. To permit insertion of the 4-kb ClaI fragment encoding pyr 5-6 at the position of the NsiI site, the existing ClaI site elsewhere in the vector was removed, and the NsiI site was converted to a ClaI site, as described below. The genomic DNA in pBluescriptSK$^-$ was digested with ClaI and KpnI, and the ends were blunted and religated to remove the ClaI site from the vector. This plasmid was then digested with NsiI, treated with calf alkaline phosphatase, and ligated with the oligonucleotide 5′ p-GTAAATCGATTTACTGCA, which anneals with itself and has phosphorylated sticky ends compatible with NsiI, as well as internal ClaI site. This construct was then subsequently digested with ClaI, treated with calf alkaline phosphatase, and ligated to the 4-kb UMP synthase gene released by digestion with ClaI of plasmid pJB1 derived from pDU3B1 (Jacquet et al., 1988). This gene replacement vector is shown in Fig. 2.

To express the 34-kD actin-bundling protein in the hygromycin-resistant 34-kD$^-$ strain, a vector was constructed which allowed expression under the control of the actin 15 promoter and actin 8 terminator (Knecht et al., 1986) using G418 as a selection marker. A 1.25-kb fragment encoding the full-length cDNA sequence of the 34-kD protein was excised from pBluescript SK$^-$/30 kD (Fechheimer et al., 1991) by digesting the plasmid with XbaI and XhoI. The fragment was blunt ended with the Klenow fragment of DNA polymerase, and was cloned into the HindIII site of pDEX RH (Faix et al., 1992), which was also blunt ended with Klenow fragment. This vector was used for transformation of the knockout cell line 34 kD$^-$/hyg to determine whether the phenotype could be reversed by expression of the 34-kD protein in the rescue cell line (34 kD$^-$/hygR).

Transformation and Isolation of Mutants

The gene replacement vector containing the hygromycin resistance cassette was used for transformation of AX2 after liberating the insert by SmaI and NotI digestion. The 34-kD expression vector containing the G418 resistance cassette was used undigested for transformation of 34 kD$^-$/hyg cells. After transformation by electroporation (Mann et al., 1994), cells were transferred to HL-5 medium, pH 7.5, and allowed to recover for 24 h. Selection was started with 10 μg/ml hygromycin B (Calbiochem/Novabiochem Corp., La Jolla, CA) or 3 μg/ml G418 (Sigma). Concentrations of hygromycin or G418 were increased stepwise to 20 or 10 μg/ml, respectively, until the control plates were cleared. Transformants were identified by colony blotting (Wallraf et al., 1986) using the 34-kD protein-specific mAb B2C (Furukawa et al., 1992).

The gene replacement vector containing the pyr 5-6 gene was used for transformation of DH1 after liberating the insert by digestion with BamHI and NotI. Transformation by electroporation was performed as described previously (Mann et al., 1994). Selection was performed by transfer to FM medium lacking uracil 24 h after electroporation. Transformants were cloned by limiting dilution, and the presence or absence of the 34-kD protein was established by Western blots.

Polymerase Chain Reaction

To prepare the *Dictyostelium* amoeba for use as a template in PCR, 1 million cells were harvested, washed two times in cold water, and suspended

in 100 μl of PCR buffer containing 50 mM KCl, 10 mM Tris, pH 9.0, and 0.1% Triton X-100. NP-40 and proteinase K were added to final concentrations of 0.5% and 100 μg/ml, respectively, and the sample was held for 45 min at 56°C and then for 10 min at 95°C.

PCR was performed in a thermal cycler (model 480; Perkin-Elmer Corp., Norwalk, CT) in 50-μl reactions containing 15 μl of processed cells or 45 ng of cloned DNA as template in the presence of 1 μM of each primer, 5 mM $MgCl_2$, and Taq polymerase, using 29 cycles of amplification using 1-min periods for denaturation, annealing, and elongation at 95°C, 51°C, and 72°C, respectively. Oligonucleotide primers 17 nucleotides in length are numbered according to the basepair sequence of the genomic clone (Fig. 1), and are designated to correspond to sense (S) or nonsense (N) orientations. Primers 1, 2, and 3 in Fig. 2 correspond to primers 147S, 812S, and 1208N, respectively.

Northern Blotting

Total RNA was isolated as described (Noegel et al., 1985) after lysis with 1% SDS in the presence of DEPC, and was purified by several phenol-chloroform extractions. For Northern blot analysis, RNA was resolved on 1.2% agarose gels in the presence of 6% formaldehyde (Sambrook et al., 1989), and was blotted onto Hybond N filters (Amersham Buchler GmbH & Co. KG, Braunschweig, Germany). Hybridization was performed at 37°C for 12–16 h in hybridization buffer containing 50% formamide plus 2× SSC. The blots were washed twice for 5 min in 2× SSC containing 0.1% SDS at room temperature, and then for 60 min in a buffer containing 50% formamide plus 2× SSC at 37°C.

Gel Electrophoresis and Western Blotting

Electrophoresis and Western blotting were performed as described previously (Laemmli, 1970; Towbin et al., 1979).

Development of Dictyostelium discoideum

Cells were grown to a density of 2–3 × 10^6 cells/ml, washed in 17 mM Soerensen phosphate buffer, pH 6.0, and resuspended at a density of 10^8 cells/ml in the same buffer. Morphology was studied by allowing 10^8 cells to develop on 1.2% (wt/vol) water agar or phosphate-buffered agar plates at 21°C. For the analysis of developmentally regulated genes, 0.75 × 10^8 cells were allowed to develop on nitrocellulose filters (Millipore type HA; Millipore, Molsheim, France) at 21°C, as described (Newell et al., 1969). Development was also examined on cells growing on SM agar plates on a lawn of *K. aerogenes* (Williams and Newell, 1976) or on nutrient agar plates on a lawn of *Escherichia coli B/2* (Noegel et al., 1985).

Pinocytosis

Pinocytosis was assessed by uptake of the fluid-phase marker lucifer yellow. Cells adherent to coverslips in chambers (Bionique Laboratories, Inc., Saranac Lake, NY) were held in HL-5 containing 1 mg/ml lucifer yellow for 15 min, followed by three quick washes in HL-5. Living AX2 and 34-kD$^-$ cells were then photographed using DIC and fluorescence optics on a microscope (IM-35; Carl Zeiss, Inc., Thornwood, NY) to monitor the uptake. Quantitative studies of pinocytosis were performed essentially as described previously (Swanson et al., 1985). Approximately 1 million cells per well were placed in 24-well plates in HL-5 growth medium and allowed to attach. At appropriate times, the solution was replaced by 0.45 ml of 0.5 mg/ml lucifer yellow (Molecular Probes, Inc., Eugene, OR) in HL-5 to permit pinocytosis. Washing was performed by submersing the plate sequentially two times in 1 liter of 17 mM Soerensen phosphate with 1 mg/ml BSA on ice, and two times in 1 liter of 17 mM phosphate on ice. The cells were then lysed by the addition of 0.5 ml of 0.05% Triton X-100 in 17 mM phosphate. Samples were taken for measurements of fluorescence and for determination of protein by the bicinchoninic acid method (Smith et al., 1985).

Immunofluorescence Microscopy

Cells were fixed, stained with rhodamine-phalloidin and DAPI, and photographed as described previously (Furukawa et al., 1994).

Measurement of Cell Size

Cells were grown to a density of 2 × 10^6 cells/ml, washed with cold Soerensen phosphate buffer, pH 6.0, resuspended to a density of 10^7 cells/ml in the same buffer in the presence of 20 mM EDTA, and shaken for 1 h at 160 rpm. This procedure led to single spherical cells. Cells were photographed, and diameters were determined from the prints. Cell size distributions were also determined using a Coulter Counter ZM (Coulter Electronics, Luton, UK).

Chemotaxis

For quantitative analysis of cell motility and chemotaxis of AX2-derived strains (AX2, 34 kD$^-$/hyg, and 34 kD$^-$/hygR), cells were grown to a density of 2–3 × 10^6 cells/ml, washed in Soerensen phosphate buffer, pH 6.0, resuspended at a density of 10^7 cells/ml, and starved for 6 h with shaking. Strain AX2 and derivatives do not require pulsing with cAMP to develop aggregation competence in shaking cultures (Beug et al., 1973). Analysis was performed using an image processing system (Segall et al., 1987) and a chemotaxis chamber (Fisher et al., 1989) with a maximum cAMP concentration of 5 × 10^{-8} M (Brink et al., 1990). Cell tracks were recorded during four 30-min periods. In each period, 40 images were taken with a time lapse of 45 s between subsequent images. During the first two half-hour periods, movement in buffer was recorded, whereas during the second two half-hour periods, a linear cAMP gradient of 2.5 × 10^{-8} M cAMP/mm was established. For analysis of AX3-derived strains (DH1 and 34 kD$^-$/ura cells), the same procedure described above was used, except that to render cells aggregation competent, pulses of cAMP (final concentration 2 × 10^{-8} M) were given every 6 min using a syringe attached to a perfusion pump.

These measurements were made on cells either on uncoated glass surfaces or on glass coated by incubation in 2 mg/ml BSA in phosphate buffer for 20 min and rinsed extensively in phosphate buffer before the addition of cells. BSA-coated glass was used, since it is a less adhesive substrate than plain glass, and since differences between wild-type and mutant cells can be more readily revealed on less adhesive substrates (Schindl et al., 1995; Weber et al., 1995).

Measurements of Filopodial Number, Length, and Branching

Cells were fixed and dehydrated on glass slides as described previously (Furukawa and Fechheimer, 1994). Coverslips were mounted onto dry specimens, which facilitated visualization of the filopodia. Images of the cells were collected using a CCD camera (model VI-470; Optronics, Inc., Galeta, CA) mounted on an inverted Diaphot microscope (Nikon Inc., Melville, NY) equipped with phase optics and a Planapo 60× lens (N.A. 1.4). Images were digitally captured and analyzed using morphometric software (IM-4000 version 3.46p; Analytical Imaging Concepts, Irvine, CA) operating on a personal computer (Gateway, N. Sioux City, SD). The filopodial lengths were obtained as pixels that were subsequently converted to micrometers by calibration with a stage micrometer. The parameters measured were the filopodial length, the number of filopodia per cell, the number of branched filopodia, and the number of branches per filopodium. The shortest distance included in all measurements was 0.4 μm.

Results

Preparation of Cells Lacking the 34-kD Protein

Genomic DNA containing sequences encoding the *Dictyostelium* 34-kD actin-bundling protein was isolated as described in Materials and Methods. The sequence reveals putative transcriptional regulatory and start sites, a coding region that is identical to that described previously from the sequence of the cDNA (Fechheimer, 1991), and an intron 233 bp in length located in the middle of the codon CAA that specifies glutamine at amino acid 15 of the polypeptide (Fig. 1). The presence of the intron was verified by PCR using the cDNA clone, genomic clone, and whole genomic DNA as templates. This 4-kb region of genomic DNA containing sequences encoding the 34-kD protein was used to prepare vectors for use in gene disruption experiments by insertion of sequences encoding resis-

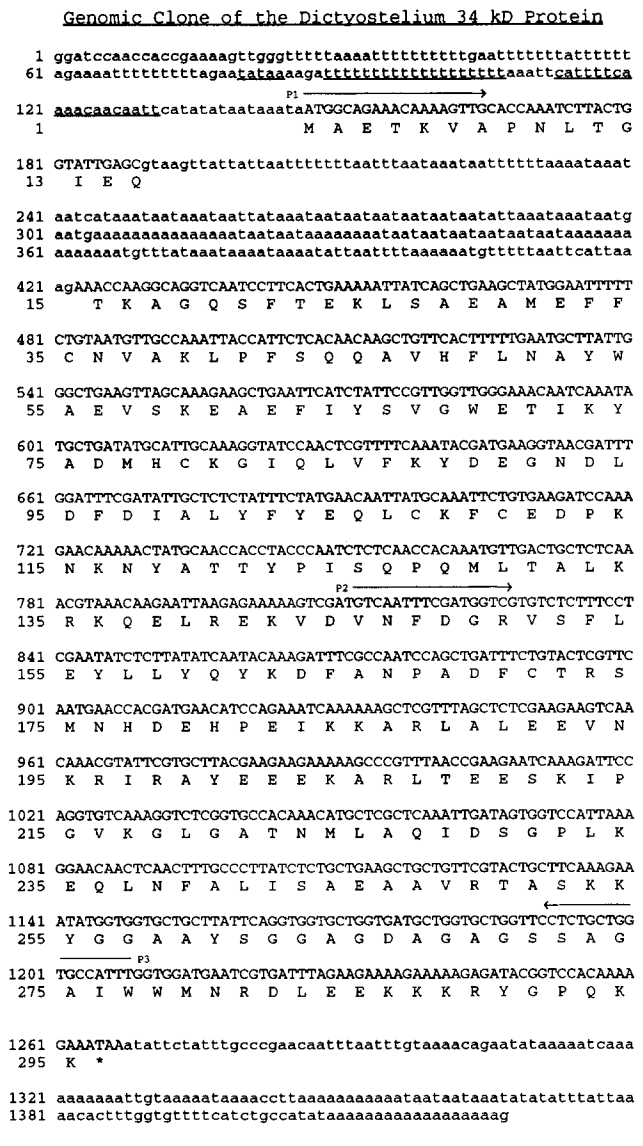

Figure 1. Sequence of the genomic DNA encoding the *Dictyostelium* 34-kD actin-bundling protein. Nucleotides 5′ to the coding sequence, intron, and 3′ to the coding sequence are lower case, while those encoding the protein are capitalized. The underlined sequences in the 5′ region are homologous to TATAA, T box, and CAAA/CAAT sequences conserved in the upstream sequences of many *Dictyostelium* genes. The intron inserted in the middle of the glutamine codon CAA at amino acid 15 contains 233 bp and consensus 5′ and 3′ splice sites. The position and orientation of oligonucleotide primers P1, P2, and P3 used for the PCR analyses are indicated by the arrows above the sequences.

tance to hygromycin (Egelhoff et al., 1989) or the ability to grow in defined medium in the absence of uracil (Jacquet et al., 1988) (Fig. 2).

Dictyostelium strains AX2 and DH1 (derived from AX3) were transformed with vectors using resistance to hygromycin and UMP synthase, respectively, and clones growing under selective conditions were analyzed. Cell lines unable to produce the 34-kD protein were obtained using both gene replacement vectors and were named 34 kD$^-$/hyg and 34 kD$^-$/ura to indicate both the selectable marker and the parent strain of *Dictyostelium* that were used. To

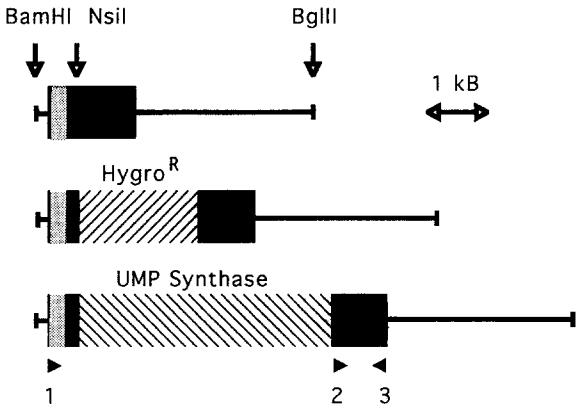

Figure 2. Vectors used for the gene replacement. The cloned genomic DNA is 4 kb in length and contains the sequences encoding the 34-kD protein and intron near the 5′ end. Regions with hatchmarks encode resistance to hygromycin (1.8 kb) and the ability to grow in the absence of uracil (4 kb) that were inserted at the NsiI site of the genomic DNA to assemble the gene replacement vectors. The intron sequence is shaded and the sequence corresponding to the cDNA is black. Restriction enzyme sites used in isolation of the genomic clone or for preparation of the gene replacement vectors are shown. Oligonucleotide primers P1, P2, and P3 used for the PCR analyses are indicated by the arrowheads.

verify that a gene replacement event had occurred, the absence of an intact copy of the gene in the 34-kD$^-$ cells was verified using a PCR assay. The presence of the 400-bp fragment using primers that do not span the selectable marker in wild-type and mutant strains verifies that portions of the gene for the 34-kD protein are present, and that the preparations are all competent to serve as templates for the PCR (Fig. 3 *A*, lanes *2*, *4*, *6*, *8*, *10*, and *12*). The presence of an ~1,060-bp PCR fragment using primers spanning the selectable marker in AX3, AX2, and DH1 wild-type cells (Fig. 3 *A*, lanes *1*, *3*, and *9*), as well as its absence in 34 kD$^-$/hyg (Fig. 3 *A*, lane *5*) and 34 kD$^-$/ura (Fig. 3 *A*, lane *11*) cells, demonstrate that an intact copy of the gene is not present in these cells.

The transcripts present in 34 kD$^-$/hyg cells were also examined. Northern blots of AX2 and 34 kD$^-$/hyg cells were probed with sequences from both the cDNA encoding the 34-kD protein and the hygromycin resistance gene (Fig. 3 *B*). AX2 cells contain an mRNA encoding the 34-kD protein of ~ 1 kb (Fig. 3 *B*, lane *2*) and no sequences encoding hygromycin resistance (Fig. 3 *B*, lane *4*). The 34 kD$^-$/hyg cells contain a truncated transcript for the 34-kD protein, and larger transcripts that contain sequences derived from both the 34-kD protein and the hygromycin resistance gene consistent with transcription across the boundary of endogenous and introduced sequences (Fig. 3 *B*, lanes *1* and *3*).

Absence of the 34-kD protein was verified by Western blots. The 34-kD protein is present in the wild-type cells AX2 and DH1 (Fig. 3 *C*, lanes *1* and *6*), but lacking in both the 34 kD$^-$/hyg and 34 kD$^-$/ura cells (Fig. 3 *C*, lanes *2* and *7*). Absence of the 34-kD protein in the 34 kD$^-$ cells was verified using mAbs IC3 and B2C, which bind to the amino- and carboxyl-terminal halves of the 34-kD protein, respec-

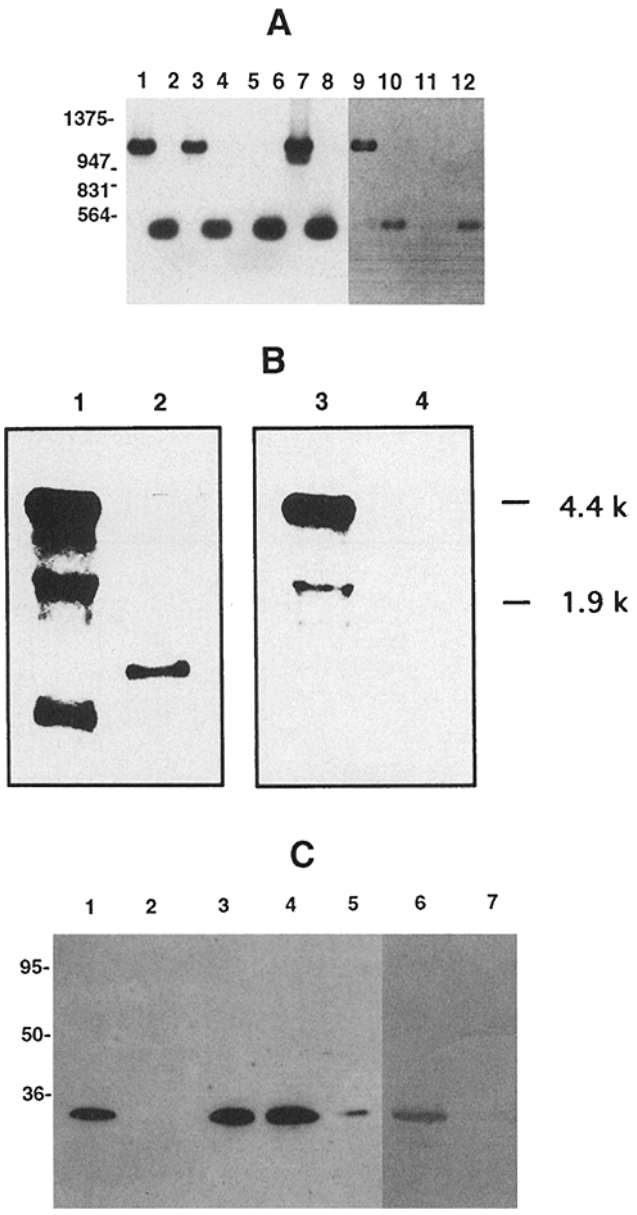

Figure 3. Changes in the DNA, RNA, and 34-kD protein result from gene replacement. (*A*) PCR of gene replacement. Oligonucleotide primers P1, P2, and P3 shown in Figs. 1 and 2 were used in combination with cloned genes or lysed *Dictyostelium* amoebae. The templates used were lysed AX3 cells (lanes *1* and *2*), lysed AX2 cells (lanes *3* and *4*), lysed 34-kD$^-$/hyg cells (lanes *5* and *6*), the purified genomic DNA encoding the 34-kD protein (lanes *7* and *8*), lysed DH1 cells (lanes *9* and *10*), and lysed 34 kD$^-$/ura cells (lanes *11* and *12*). The combinations of primers 1 and 3 (lanes *1*, *3*, *5*, *7*, *9*, and *11*) or primers 2 and 3 (lanes *2*, *4*, *6*, *8*, *10*, and *12*) are expected to produce fragments of 1,060 or 400 bp from the intact gene, respectively. Both fragments were observed using either wild-type cells or the genomic clone as template. The 1,060-bp PCR fragment is not observed in the 34-kD$^-$/hyg cells (lane *5*) or the 34-kD$^-$/ura cells (lane *11*), indicating that an intact copy of the gene for the 34-kD protein is not present. (*B*) Northern blots of gene replacement. Northern blot analysis of RNA from wild-type AX2 (lanes *2* and *4*) and 34 kD$^-$/hyg cells (lanes *1* and *3*). Total RNA was resolved, blotted, and hybridized as described in Materials and Methods. 10 μg of RNA was loaded per lane. The blot was probed with a 34-kD specific cDNA (lanes *1* and *2*), and after stripping, with a probe corresponding to the hygromycin gene (lanes *3* and *4*). Markers correspond to the *Dictyostelium* ribosomal RNA bands. (*C*) Western blot of gene replacement. Lanes were loaded with a homogenate of the cell type indicated, transferred to nitrocellulose, and stained with mAb B2C elicited against the 34-kD protein. Lane *1*, AX2; lane *2*, 34 kD$^-$/hyg; lane *3*, 34 kD$^-$/hygR1; lane *4*, 34 kD$^-$/hygR2; lane *5*, 34 kD$^-$/hygR4; lane *6*, DH1; lane *7*, 34 kD$^-$/ura. The 34-kD protein is present in wild-type cells AX2 and DH1 (lanes *1* and *6*), lacking in 34 kD$^-$/hyg and 34 kD$^-$/ura cells (lanes *2* and *7*), and present in the rescue strains in which the 34-kD protein has been reexpressed (lanes *3–5*).

tively (Lim, R.W.L., and M. Fechheimer, unpublished data). The Western blots indicate that protein products that might result from translation of the truncated transcripts (Fig. 3 *B*) do not accumulate. These results confirm the replacement of the gene encoding the 34-kD protein at the level of the DNA, RNA, and protein.

Growth, Size Distribution, and Endocytosis of the 34 kD$^-$ Strain

Whereas naturally living *D. discoideum* feed on bacteria, some strains adapted to laboratory conditions are also able to grow in axenic cultures. Since cytoskeletal proteins play a role in endocytosis, growth rates were determined under several conditions. Cells lacking the 34-kD protein can grow normally in suspension. Growth curves for cells in shaking cultures in HL-5 reveal a doubling time of 11 h for both 34 kD$^-$/hyg and AX2 wild-type cells (Fig. 4 *A*). Similarly, the rate of growth in shaking suspension cultures of DH1 and 34 kD$^-$/ura cells did not differ significantly with a doubling time of 12.5 h (Fig. 4 *B*). Examination of suspension-grown cells after staining with DAPI revealed no increase in multinucleated cells (data not shown). Thus, there is no evidence for a defect of cytokinesis in the 34-kD$^-$ cells. The cell size distribution of 34 kD$^-$/hyg and AX2 wild-type cells grown in suspension was determined from prints, as described in Materials and Methods. The average diameter was 11.32 ± 2.1 and 10.61 ± 1.67 μm for AX2 and 34 kD$^-$/hyg, respectively. This difference is not significant according to the Student's *t* test. A Coulter Counter was used to provide a measurement of the cell size in suspension, indicating that cell size is similar for the AX2 and 34 kD$^-$/hyg cells (data not shown). These results confirm that growth, division, and volume regulation are normal in the absence of the 34-kD protein.

For the determination of growth rates in bacterial suspension, 5 ml of 17 mM Soerensen phosphate buffer, pH 6.0, containing 10^{11} *E. coli B/r* bacteria were inoculated with 5×10^4 cells/ml of AX2 wild-type or 34 kD$^-$/hyg *Dictyostelium.* Cell counts were determined every 3 h until clearing of the bacterial suspension occurred. Growth curves show a doubling time of 2.6 h for both AX2 and 34 kD$^-$/hyg cells (Fig. 4 *C*). Furthermore, growth rates on SM-agar plates with bacteria as a food source were also similar for both strains (Fig. 4 *D*). Overall, the unaltered doubling times of the 34 kD$^-$/hyg mutant, as compared to AX2 wild-type in axenic medium and in the presence of bacteria, are an indication that pinocytosis and phagocytosis are not significantly impaired.

The ability of the cells lacking the 34-kD protein to perform pinocytosis was examined directly by study of the

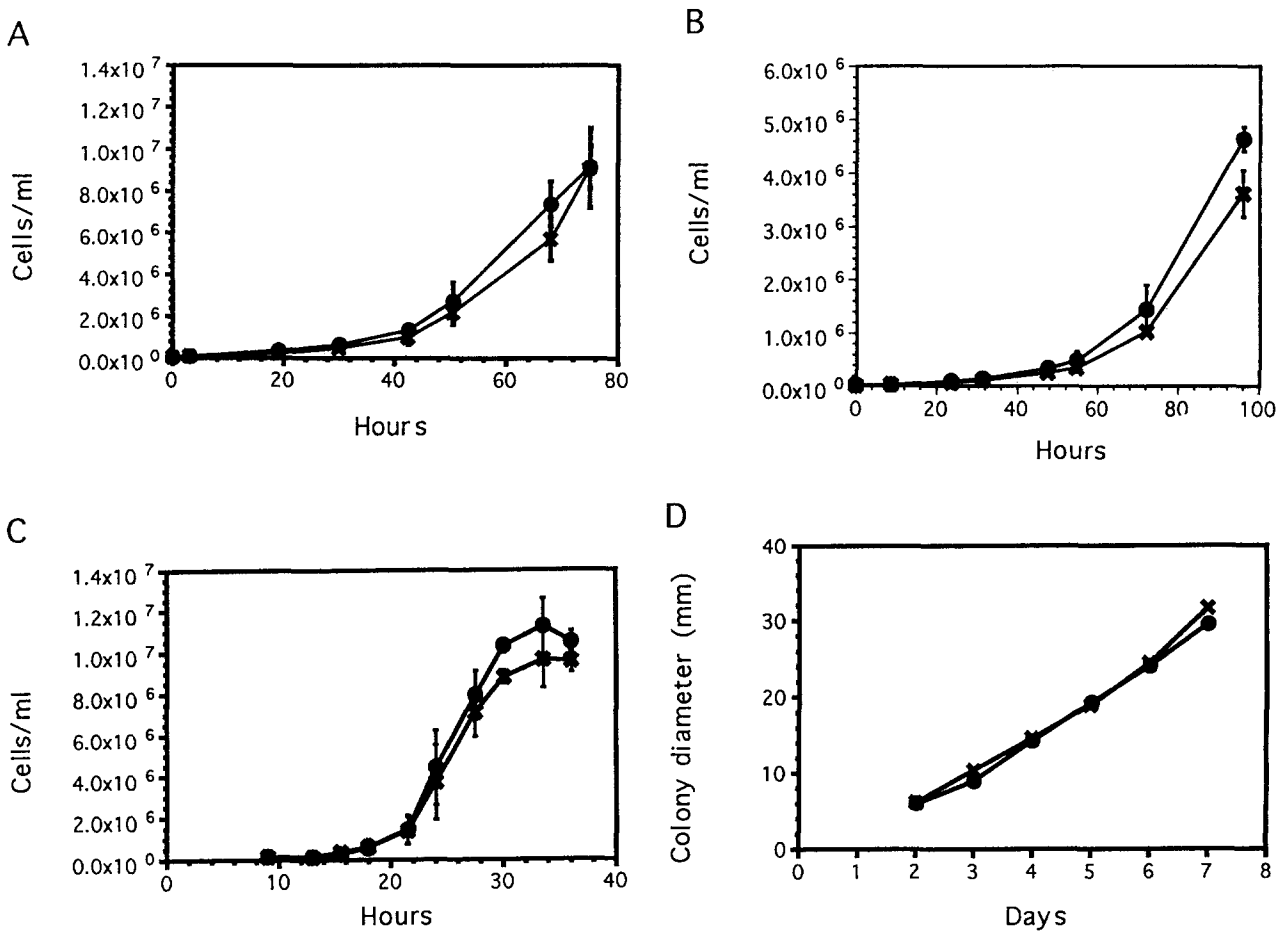

Figure 4. Growth of wild-type and 34 kD$^-$ cells. (*A*) Growth in axenic suspension cultures. AX2 (6) and 34 kD$^-$/hyg (●) cells were seeded in HL-5 at a density of 5×10^4 cells/ml and grown with shaking. Cells were counted at the times indicated. Results are the average ± SD of six determinations. Both wild-type and 34 kD$^-$/hyg cells grow with a doubling time of 11 h. (*B*) Growth in axenic suspension cultures. Experiments were performed essentially as described in *A*, except that growth of the parental strain DH1 (6) was compared to 34 kD$^-$/ura (●) in HL-5 after seeding at 2×10^4 cells/ml. The doubling time of these cultures was 12.5 h. (*C*) Growth in suspension cultures with *E. coli* as a food source. Suspension cultures of AX2 (6) and 34 kD$^-$/hyg (●) cells were prepared in phosphate buffer with *E. coli* as the food source, and the cell density was monitored as a function of time. AX2 and 34 kD$^-$ cells grew with a doubling time of 2.6 h. (*D*) Growth on agar with bacteria as a food source. AX2 (6) and 34 kD$^-$/hyg (●) were plated at limiting dilution on nutrient agar with bacteria so that clones arose from growth of single cells. Colony diameter was recorded as a measure of the growth rate. AX2 and 34 kD$^-$/hyg exhibit identical growth rates under these conditions.

ability to endocytose the fluid-phase marker lucifer yellow using both fluorescence microscopy and spectrofluorometry to observe and quantify the endocytosed probe. The number and distribution of lucifer yellow endosomes in AX2 and 34 kD$^-$/hyg are similar by fluorescence microscopy (Fig. 5 *A*). The quantitative studies of the uptake of lucifer yellow confirm that 34 kD$^-$/hyg and 34 kD$^-$/ura cells have no deficiency in the accumulation of bulk fluid phase, as compared to AX2 and DH1 wild-types, respectively (Fig. 5 *B*).

Development of the 34-kD$^-$ Cells

Upon starvation, *D. discoideum* cells enter a developmental cycle that leads from single amoebae to the formation of a multicellular fruiting body. This cycle involves differentiation into at least two cell types, prespore and prestalk cells, which sort to give rise to a mature fruiting body consisting of stalk and spore cells. This process requires locomotion and chemotaxis by single cells, as well as morphogenesis, pattern formation, and motility of multicellular structures. To test if a deficiency of the 34-kD protein causes any alteration in the developmental pattern of *D. discoideum*, development of AX2 and 34 kD$^-$/hyg cells, as well as DH1 and 34 kD$^-$/ura cells, was analyzed under several conditions. Visual inspection of cells starved on phosphate-buffered agar and on water-agar, as well as of cells growing on *K. aerogenes*, did not reveal any difference between wild-type and 34 kD$^-$/hyg cells in the formation of morphogenetic structures. To analyze for more subtle differences in the rate or quality of development, the timing of expression of developmentally regulated genes was compared. For this purpose, AX2 or 34 kD$^-$/hyg cells were allowed to develop synchronously on nitrocellulose filters, and RNA was isolated at different stages. As probes for Northern blots, cDNAs coding for the cAMP receptor

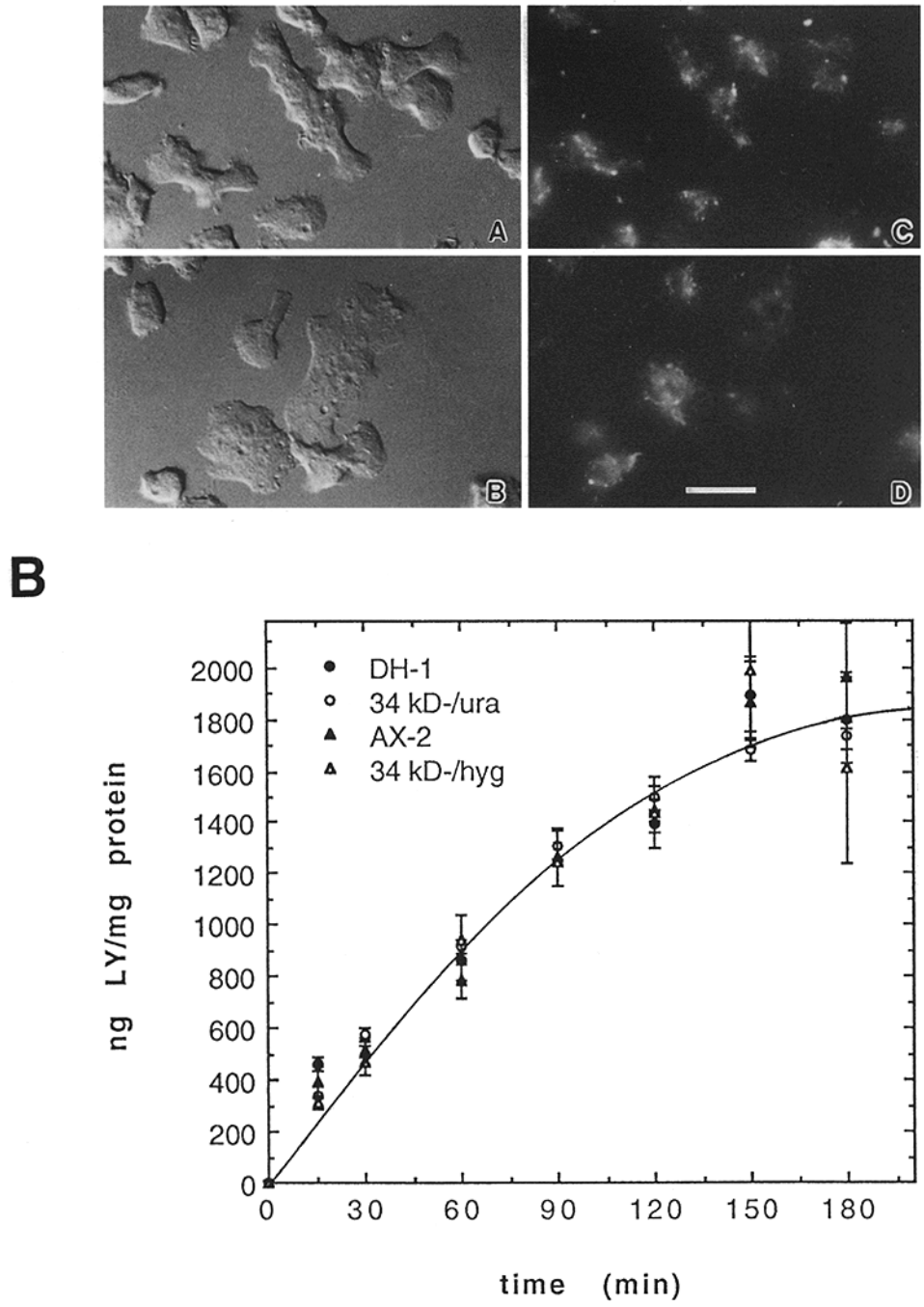

Figure 5. Fluid phase uptake. (*A*) Qualitative examination of pinocytosis by AX2 and 34 kD$^-$/hyg cells. Cells adherent to coverslips were held in medium containing 1 mg/ml lucifer yellow for 15 min, washed, and examined by differential interference contrast (*A* and *B*) and fluorescence (*C* and *D*) microscopy. Fluid-phase pinocytosis by AX2 (*A* and *C*) and 34 kD$^-$/hyg (*B* and *D*) cells is not significantly different. Bar, 10 μm. (*B*) Quantitative examination of pinocytosis by wild-type and 34 kD$^-$ cells. AX2 (▲), 34 kD$^-$/hyg (△), DH1 (●), and 34 kD$^-$/ura (○) cells adherent to 24-well dishes were allowed to pinocytose lucifer yellow for various periods of time. Results are the average of triplicate determinations ± SD of nanograms of lucifer yellow per milligrams of cell protein. Data points for which the error bars are not visible in the figure have standard deviations that are not larger than the symbol. Fluid-phase pinocytosis by wild-type and 34 kD$^-$ cells is not significantly different. Similar results were obtained in independent experiments.

cARI (Klein et al., 1988), the prespore-specific protein psA encoded by the *D19* gene (Early et al., 1988), and the prestalk-specific extracellular matrix proteins ecmA and ecmB encoded by the *D56* and *D63* genes, respectively, were used (Jermyn et al., 1987). The message for *cARI*, detectable at low levels in growing cells of both strains, increases after the onset of starvation (Fig. 6). After aggregation, a series of other *cARs* are expressed, which, because of the high degree of homology, are detected with the cDNA probe for *cARI*. Overall, substantial differences in the timing and level of expression of the genes tested were not observed, indicating that the absence of the 34-kD protein results in no detectable alterations in the differentiation and morphogenesis in *D. discoideum*.

Chemotaxis and Motility

Cell motility and chemotactic orientation in the presence or absence of a linear gradient of cAMP were quantitatively analyzed using a chemotaxis chamber and a computer-controlled image processing system (Table I). The rate of locomotion of both the wild-type AX2 and the 34 kD$^-$/hyg

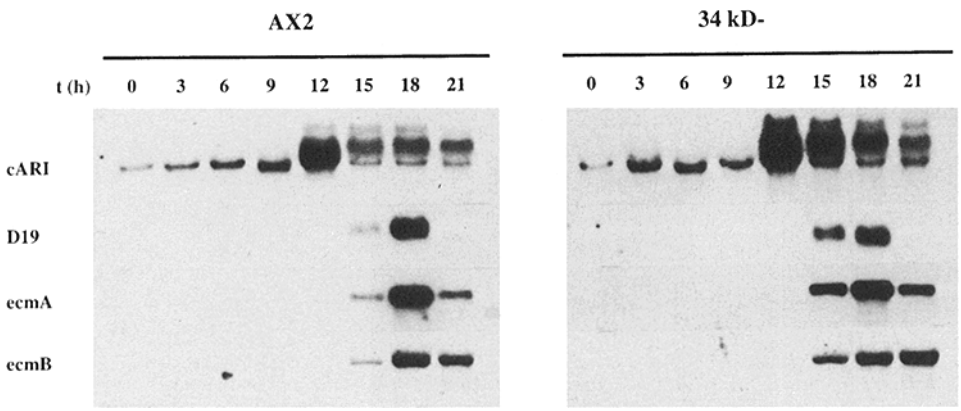

Figure 6. Expression of developmentally regulated genes in wild-type (AX2) and 34 kD$^-$/hyg cells. 0.75×10^8 cells were allowed to develop on nitrocellulose filters at 21°C for the times indicated in hours. Cells were washed off the filters and total RNA was isolated. 10 μg RNA per time point were resolved by gel electrophoresis and transferred to membranes for Northern analysis with the corresponding probes. Changes in steady-state levels of mRNAs encoding developmentally regulated genes are similar in magnitude and timing in AX2 and 34 kD$^-$/hyg cells.

cells was 7–8 μm/min in the absence of a chemotactic gradient, and ~12 μm/min in the presence of a cAMP gradient on either uncoated or BSA-coated glass. No differences in the rate of locomotion or in the capability to orient in the cAMP gradient between the two strains were apparent. The persistence of motility was assessed by measurement of the average turn of the cells between successive images. AX2 and 34 kD$^-$/hyg cells had similar persistence of motility on uncoated glass. The 34-kD$^-$/hyg cells, however, had a significantly lower average turn, indicating a higher persistence of motility in either the presence or absence of a cAMP gradient on BSA-coated glass.

The AX3-derived strains DH1 and the 34-kD$^-$/ura mutant were analyzed only on BSA-coated glass surfaces. DH1 and 34 kD$^-$/ura exhibit a similar rate of locomotion and the ability to orient in a chemotactic gradient. The 34 kD$^-$/ura cells exhibited a higher persistence of motility than DH1 during migration in the absence of a chemotactic gradient.

Differences in motility between parental *Dictyostelium* strains were also noted. The AX3-derived strains DH1 and 34 kD$^-$/ura exhibit a somewhat lower rate of locomotion in the presence of a cAMP gradient, as compared to AX2 and the 34-kD$^-$/hyg strains, respectively. DH1 cells also showed a lower average turn than AX2 in the presence of a chemotactic gradient.

Changes in the Filopodia in Cells Lacking the 34-kD Protein

The appearance of the 34-kD$^-$ cells was compared to wild-type using DIC microscopy and rhodamine phalloi-

Table I. Motility and Chemotactic Orientation of Wild-type and Mutant Strains of This Study

Glass	Condition	Strain	Speed	Orientation	Average turn
			μm/min	*cos θ*	
Uncoated	Buffer	AX2	7.36 ± 1.59	−0.051 ± 0.140	47.53 ± 6.91
		34 kD$^-$hyg	7.14 ± 2.46	−0.077 ± 0.102	48.10 ± 8.56*
	Gradient	AX2	12.29 ± 2.14	0.188 ± 0.048	40.81 ± 4.91
		34 kD$^-$/hyg	12.17 ± 1.05	0.176 ± 0.071	36.38 ± 3.17*
BSA coated	Buffer	AX2	8.18 ± 1.96	−0.030 ± 0.053	43.63 ± 3.14
		34 kD$^-$/hyg	7.72 ± 2.64	−0.034 ± 0.086	31.36 ± 2.89‡
		34 kD$^-$/hygR1	8.36 ± 0.85	0.002 ± 0.048	47.21 ± 4.19§
		34 kD$^-$/hygR4	10.49 ± 1.61	0.043 ± 0.068	41.94 ± 3.76§
	Gradient	AX2	12.16 ± 2.53	0.246 ± 0.056	39.35 ± 3.89
		34 kD$^-$/hyg	12.47 ± 1.17	0.242 ± 0.048	29.95 ± 2.95‡
		34 kD$^-$/hygR1	12.54 ± 1.32	0.176 ± 0.058	37.19 ± 2.87§
		34 kD$^-$/hygR4	11.79 ± 0.76	0.246 ± 0.085	32.78 ± 3.92‡
	Buffer	DH1	9.25 ± 1.45	−0.013 ± 0.047	45.78 ± 6.29
		34 kD$^-$/ura	6.55 ± 1.77	0.003 ± 0.062	35.94 ± 2.46‖
	Gradient	DH1	9.43 ± 1.40‡	0.201 ± 0.040	33.16 ± 2.31‡
		34 kD$^-$/ura	7.57 ± 1.26§	0.221 ± 0.051	28.47 ± 2.15

Cell tracks were recorded during four 30-min periods, as described in Materials and Methods. Speed, orientation, and average turn were calculated for the second (buffer) and fourth (gradient) half-hour periods. Orientation was calculated as the ratio between the distance from original to final position and the total path length, multiplied by the cosine of the angle that forms the track of the cell with the direction of the gradient. Average turn is the average change of direction (in degrees) between two subsequent images. Data are mean ± SD of 5–10 independent experiments. The number of cells recorded in each experiment ranged from 40 to 200. $P < 0.005$ was considered significant (ANOVA). Only relevant statistical comparisons are shown.

*Significant relative to the same strain on BSA-coated glass.

‡Significant relative to AX2.

§Significant relative to 34 kD$^-$/hyg.

‖Significant relative to DH1.

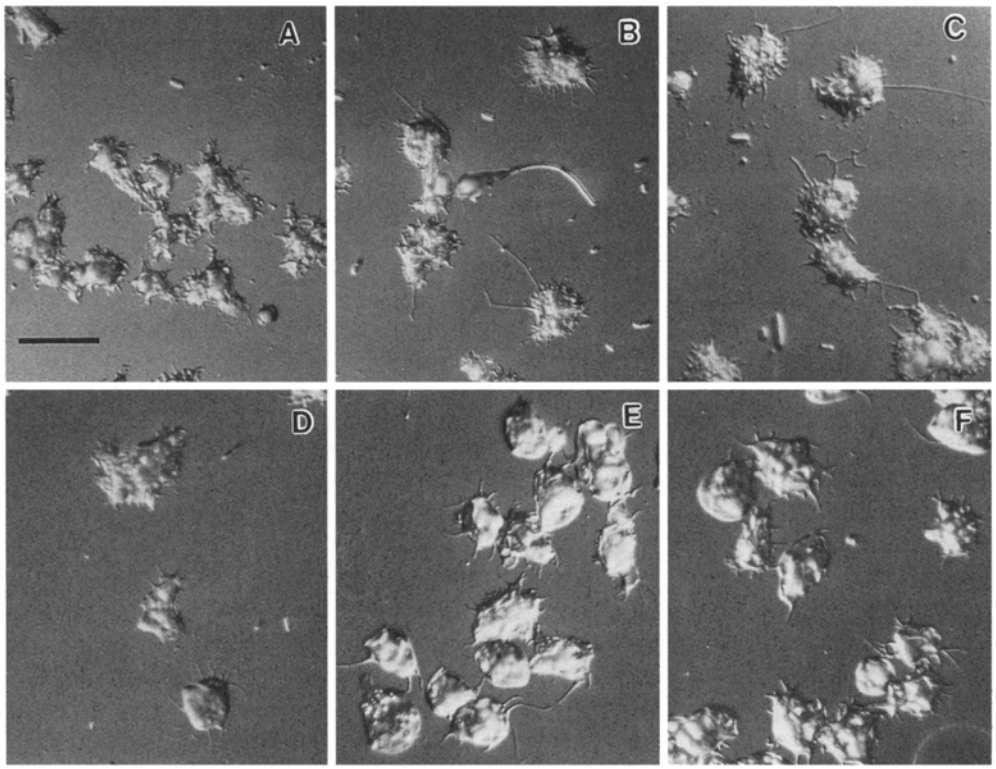

Figure 7. Morphology of filopodia. Wild-type, 34 kD$^-$, and rescue cells were placed on glass coverslips in 17 mM Soerensen's phosphate buffer, allowed to adhere and spread, fixed, and examined for the presence of filopodia by DIC microscopy. (*A*) AX2, (*B* and *C*) 34 kD$^-$/hyg, (*D*) 34 kD$^-$/hygR1, (*E*) DH1, and (*F*) 34 kD$^-$/ura. The 34-kD$^-$/hyg cells appear to exhibit more long and branched filopodia than do wild-type cells. The differences are not apparent in the rescue strain. The 34-kD$^-$/ura cells have shorter and more abundant filopodia than DH1. Bar, 20 μm.

din staining to localize F-actin. Branched filopodia and long filopodia appeared more prominent in 34 kD$^-$/hyg than in AX2 cells (Figs. 7, *A–C*, and 8, *A* and *B*). The filopodia were analyzed quantitatively for number per cell, branching, and length to examine this aspect of cell morphology in detail. There were ~2.5 filopodia per cell in AX2 and 34 kD$^-$/hyg, and the values were not significantly different (Table II). The 34 kD$^-$/hyg cells were found to contain more filopodia with extensive branching patterns (Fig. 9). These differences were significant ($P < 0.01$) when compared using the Mann-Whitney test, a nonparametric counterpart of the Student's *t* test (Gibbons, 1985). Filopodial length was then examined in two ways. The median filopodial lengths of AX2 and 34 kD$^-$/hyg were first calculated so that each segment in a branched filopodial structure was treated as a separate filopodium. The values are not significantly different using the Mann-Whitney test. Since the 34-kD$^-$/hyg cells contain significantly more filopodia in branched structures than AX2 (Fig. 9), the total length contained in filopodia (calculated as the sum of all segments of a filopodium including branches) was then determined so that the length distributions of the filopodia of wild-type and mutant strains could be compared (Fig. 10, *A* and *B*). In addition, the median and quartile values of these distributions were determined (Table II). The Mann-Whitney test shows that the filopodia of 34 kD$^-$/hyg are significantly longer than AX2 ($P < 0.01$). The Siegel-Tukey test, a nonparametric test that determines whether the range of two populations is significantly different (Gibbons, 1985), was used to show that the length of the filopodia of the 34-kD$^-$/hyg cells is significantly skewed towards longer lengths, as compared to AX2 ($P < 0.02$).

Filopodia of the 34-kD$^-$/ura line were also found to differ from the parental line DH1 (Figs. 7, *E* and *F*, and 8, *C* and *D*). Length distributions of filopodia on 34 kD$^-$/ura appeared shorter than those of DH1 (Fig. 10, *C* and *D*). Analysis of the median and quartile values shows that the parental DH1 differ from the 34-kD$^-$/ura cells in having more of the longest filopodia (Table II). These differences are significant by the Mann-Whitney and Siegel-Tukey tests ($P < 0.01$ for both). In addition, the 34-kD$^-$/ura cells have significantly more filopodia per cell than DH1 (Fig. 11). The median values are 3 and 15 filopodia per cell for DH1 and 34 kD$^-$/ura cells, respectively. These differences are significant according to the Mann-Whitney test ($P < 0.01$). Branching of filopodia does not differ considerably between DH1 and 34 kD$^-$/ura, since branched filopodia are <10% of all filopodia in both strains (data not shown).

It was also noted that the 34-kD$^-$/hyg cells exhibit some lack of coordination of motility, since these cells are more likely than the wild-type cells to shed bits of cytoplasm while migrating on a glass substrate. Examination of the 34-kD$^-$/hyg cells by light microscopy and rhodamine-phalloidin staining to localize F-actin reveals the broken pieces of cytoplasm (Fig. 12, *C–H*). This shedding was occasionally observed in AX2 (Fig. 12, *A* and *B*), but was significantly more abundant in the 34-kD$^-$/hyg cells. Visual inspection of the 34-kD$^-$/hyg cells revealed that protrusion of extensions was normal, but that retraction was deficient, suggesting a mechanism for the shedding. These results reveal a remarkable loss of the ability to control and regulate cell movements.

Changes in abundance of other actin cross-linking proteins in the 34-kD$^-$/hyg cells was also examined to determine whether compensatory changes might be apparent. Significant differences in the content of α-actinin and the

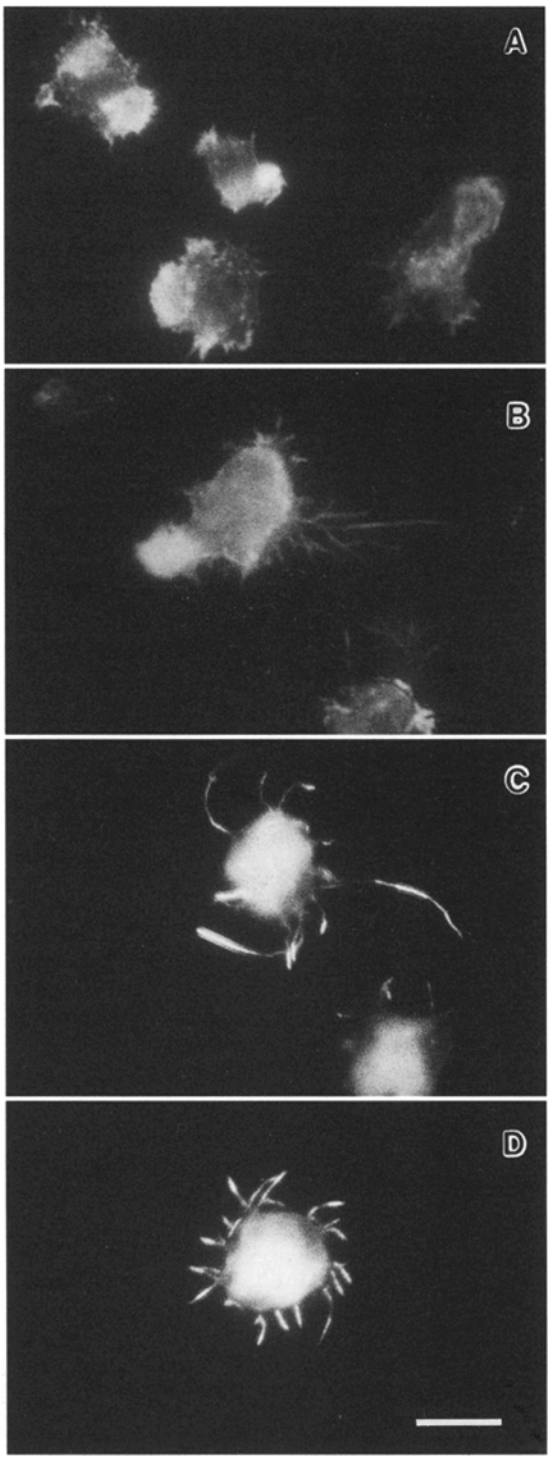

Figure 8. F-actin distribution of wild-type and 34 kD$^-$ cells. Wild-type and 34 kD$^-$ cells were placed on glass coverslips in 17 mM Soerensen's phosphate buffer, allowed to adhere and spread, fixed, and then stained with rhodamine phalloidin to localize F-actin. (*A*) AX2, (*B*) 34 kD$^-$/hyg, (*C*) DH1, and (*D*) 34 kD$^-$/ura. Bar, 10 μm.

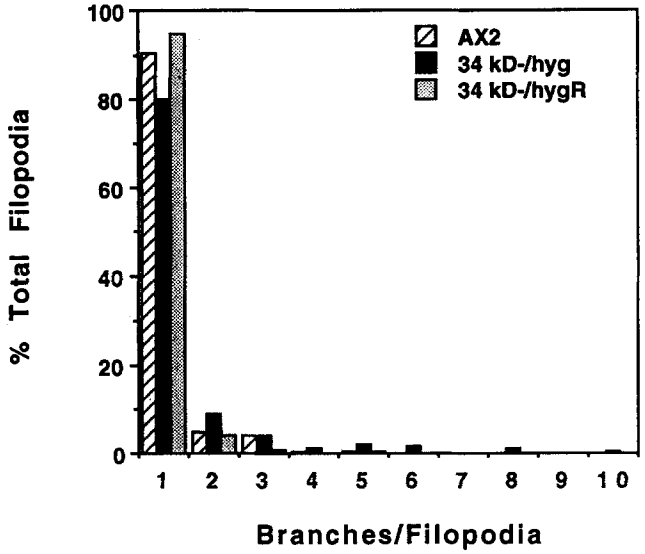

Figure 9. Branching of filopodia. The frequency of filopodia with the indicated number of branched structures was measured as described in Materials and Methods. Highly branched filopodia are prominent in 34 kD$^-$/hyg, but not in AX2 or 34 kD$^-$/hygR1.

gelation factor (ABP 120) between wild-type and 34 kD$^-$/hyg cells were not detected (data not shown).

Rescue of Mutants by Expression of the 34-kD Protein

The 34-kD$^-$/hyg cells were transformed with a plasmid that allowed the expression of the 34-kD actin-bundling protein under the control of actin 15 promoter and actin 8 terminator, and transformants were screened for the expression of the protein. Two clones, 34 kD$^-$/hygR1 and 34 kD$^-$/hygR2, expressed the 34-kD protein at levels comparable to that of the wild-type AX2 (Fig. 3 *C*, lanes *3* and *4*). A third clone, 34 kD$^-$/hygR4, expressed the protein at levels ~10% of wild type (Fig. 3 *C*, lane *5*).

Cell motility and chemotactic orientation were analyzed in the rescue mutants and compared to the AX2 and 34 kD$^-$/hyg (Table I). The behavior of the rescue strain 34 kD$^-$hyg/R1 was not significantly different from AX2, and it differed significantly from mutant 34 kD$^-$/hyg in the average change of direction in the presence or absence of a gradient of the chemoattractant cAMP (Table I). Mutant 34 kD$^-$/hygR4, which expresses low levels of the 34-kD protein, showed an average turn frequency close to that of AX2 cells in the absence of a cAMP gradient, but similar to that of 34 kD$^-$/hyg cells in the presence of a cAMP gradient, indicating that the levels of protein expressed are not high enough to completely rescue the phenotype of 34 kD$^-$/hyg cells.

Filopodia in the 34-kD$^-$/hygR1 strain were similar to AX2, as examined by light microscopy (Fig. 7 *D*). The length distribution of the filopodia of the 34-kD$^-$/hygR1 strain was similar to AX2 (Table II). Analyses by the Mann-Whitney test shows that AX2 is not significantly different from 34 kD$^-$/hygR, and that 34 kD$^-$/hyg is significantly different from 34 kD$^-$/hygR with regard to filopodial length ($P < 0.01$). Similarly, branching of filopodia of the 34-kD$^-$hyg/R1 strain is not significantly different than

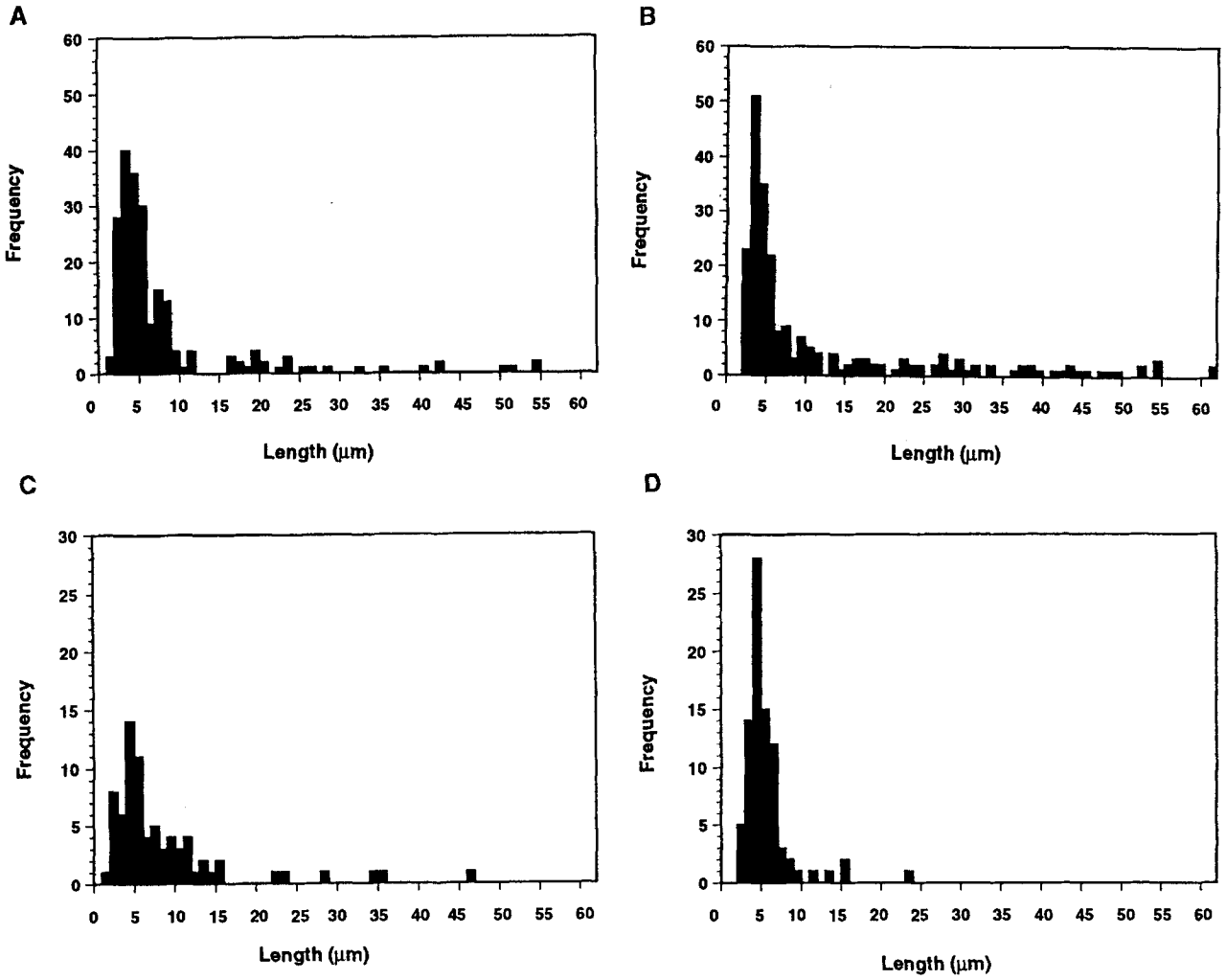

Figure 10. Length of filopodia on wild-type and 34 kD$^-$ cells. Histograms of the frequency of filopodia with indicated length distributions were measured for (*A*) AX2, (*B*) 34 kD$^-$/hyg, (*C*) DH1, and (*D*) 34 kD$^-$/ura. Filopodia were measured as described in Materials and Methods. The 34-kD$^-$/hyg cells have a longer length distribution than AX2, while the filopodia of 34 kD$^-$/ura are shorter than the parental strain DH1.

AX2, and it differed significantly from 34 kD$^-$/hyg (Fig. 9, Mann-Whitney $P < 0.03$).

Discussion

Actin-binding proteins are believed to play key roles in cell structure and movement, as regulators of the assembly, stability, and localization of actin filaments in the cytoplasm. The multiple actin cross-linking proteins in *Dictyostelium* are abundant, localized at sites of cell movements and highly conserved across the eukaryotes, implying a significant role in cell shape and motility. Thus, the ability to test function directly by targeted gene disruption has been a key feature of the *Dictyostelium* system that has been exploited in recent years.

Cells lacking α-actinin were reported to exhibit no defects in any aspects of cell structure, movement, or differentiation (Witke et al., 1987; Schleicher et al., 1988). Cells lacking the gelation factor (ABP 120) derived from strain AX2 by chemical mutagenesis were reported to locomote, chemotax, and complete development normally (Brink et al., 1990), while cells lacking the gelation factor derived from strain AX3 exhibit reduced rates of motility, alterations in pseudopod morphology, actin filament organization, and phagocytosis (Cox et al., 1992, 1995, 1996). The difference clearly results from the strain of *Dictyostelium* and not from the method of preparation of the mutants (Rivero et al., 1996). Cell lines deficient in both α-actinin

Table II. Characterization of Filopodial Length Distributions

Strain	First quartile	Median	Third quartile	No. of cells	No. of filopodia
AX2	2.53	3.98	6.84	88	212
34 kD$^-$/hyg	2.58	4.23	13.39	90	242
34 kD$^-$/hygR	2.39	3.67	4.98	47	315
DH1	2.68	4.42	8.50	67	276
34 kD$^-$/ura	2.39	3.48	4.74	45	342

All lengths are in micrometers.
Filopodial lengths for AX2, 34 kD$^-$/hyg, and 34 kD$^-$/hygR are calculated from the total length of all filopodia and branches.
Filopodial lengths for DH1 and 34 kD$^-$/ura are calculated without summation of branches.

and the gelation factor derived from strain AX2 cannot complete multicellular development (Witke et al., 1992; Rivero et al., 1996). These findings provide a strong case for the concept of functional redundancy. That is, cells lacking either α-actinin or the gelation factor could complete development, while cells lacking both were arrested after formation of a cell aggregate termed the mound. Additional data are required to further elucidate the functions of the multiple actin cross-linking proteins and the unique or redundant contributions that they make to control of cell shape and movement.

The 34,000-D protein is a prominent actin cross-linking protein of *Dictyostelium* whose function has been assessed using the gene replacement approach in the present study. It was predicted that cells lacking the *Dictyostelium* 34-kD protein would exhibit a defect in cytokinesis, since the protein is found in the cleavage furrow (Furukawa and Fechheimer, 1994). In addition, defects in phagocytosis were anticipated, since the protein is localized in the phagocytic cup, and since loading of cells with antibodies reactive with the 34-kD protein results in a statistically significant inhibition of phagocytosis (Furukawa et al., 1992; Furukawa and Fechheimer, 1994). Finally, it was predicted that cells lacking the 34-kD protein would exhibit defects in multicellular development, since this protein is localized at sites of cell–cell contacts and is associated with membranes isolated from cell contact regions (Fechheimer et al., 1994).

A singular feature of the results of our investigations of cells lacking the *Dictyostelium* 34-kD protein is that none of our simple predictions have proved to be correct. First, cells lacking the 34-kD protein complete cytokinesis normally, even in suspension, and do not accumulate multiple nuclei, as has been observed for cells defective in cytokinesis because of an absence of myosin II (de Lozanne and Spudich, 1987; Knecht and Loomis, 1987), coronin (de Hostos et al., 1993), or the profilins (Haugwitz et al., 1994). Second, both pinocytosis and phagocytosis by the cells lacking the 34-kD protein appear normal. Finally, development, morphogenesis, and gene expression by cells lacking the 34-kD protein show no significant differences from wild type.

The investigations did reveal some significant differences attributable to the lack of the 34-kD protein. First, *Dictyostelium* amoebae lacking the 34-kD protein derived from both parental strains AX2 and AX3 exhibit a higher persistence of motility, assessed as a decrease in the average angle of deviation of the direction of cell movement between successive images recorded at 45 s intervals (Table I). Neither the rate of cell locomotion nor the ability to orient in the gradient of chemoattractant revealed any significant difference between wild-type and 34 kD$^-$ cells. This difference in persistence of motility could only be observed when cells were deposited on BSA-coated glass. This result is in agreement with recent reports by Schindl et al. (1995) and Weber et al. (1995). Using reflection interference contrast microscopy on AX2 and cytoskeletal mutants, these authors have shown that phenotypic changes associated with the lack of actin-binding proteins are most obvious on weakly adherent surfaces. Strongly adherent substrates, like uncoated glass, can compensate for defects in the stability of newly formed pseudopods and in the capability of forming contacts to the surface.

Second, cells lacking the 34-kD protein differed from wild-type cells in the number and length of filopodia, but the nature of the difference was dependent on the strain of *Dictyostelium* that was used. The 34-kD$^-$/hyg cells had more long filopodia than their parental AX2 cells (Figs. 7, 8, and 10 and Table II) and more branching of filopodia (Fig. 9), but the number of filopodia per cell was not significantly changed. The 34-kD$^-$/ura cells had slightly shorter filopodia than the parental cell DH1 (Figs. 7, 8, and 10 and Table II), a uracil requiring derivative of AX3, and were characterized primarily by an increase in the number of filopodia per cell (Fig. 11). The 34-kD protein is present in filopodia, and has been proposed to mediate formation or disassembly of these structures in response to dynamic changes in the free calcium ion concentration (Fechheimer, 1987; Furukawa and Fechheimer, 1990). The results indicate a clear change in the filopodial dynamics whose outcome differs in the two strains.

Third, the 34 kD$^-$/hyg cells have a tendency to shed bits of cytoplasm on a glass substrate. This behavior is observed even for the parent cell line AX2 on the highly adhesive substrate uncoated glass (Schindl et al., 1995), but is more prominent in the 34 kD$^-$/hyg cells (Fig. 12). The greater tendency to shed cytoplasm may be related to the increased length (Table II) and branching (Fig. 9) of filopodia in the 34-kD$^-$/hyg cells, since these extended structures may be more likely to fracture because of a failure to retract. In addition, this behavior may reflect a lack of temporal and spatial coordination of motility and/or cytoplasmic organization. This behavior is consistent with an inability to regulate gel/sol transformations that have been implicated in cell movements through a coupling of solation with contraction (Taylor and Fechheimer, 1982; Janson et al., 1991).

It is striking that all three of the observations—aberrant filopodia, shedding of blebs of cytoplasm, and increased persistence of motility—could also be related to a deficiency in the ability to reorganize regions of cytoplasm. This common feature might suggest that gelled or bundled actin structures of wild-type cells, normally cross-linked by the 34-kD protein, are more readily reorganized than those present in the 34-kD$^-$ cells. This greater persistence

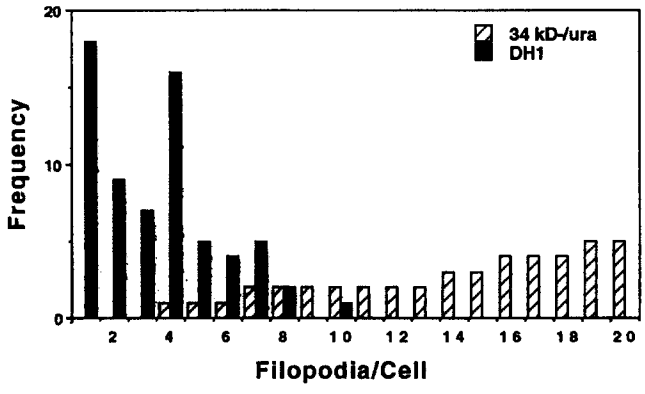

Figure 11. The number of filopodia per cell in wild-type and 34 kD$^-$/ura strains. The frequency of cells with the indicated number of filopodia per cell was measured for DH1 and 34 kD$^-$/ura, as described in Materials and Methods. The 34-kD$^-$/ura cells have more filopodia per cell than the DH1 wild type.

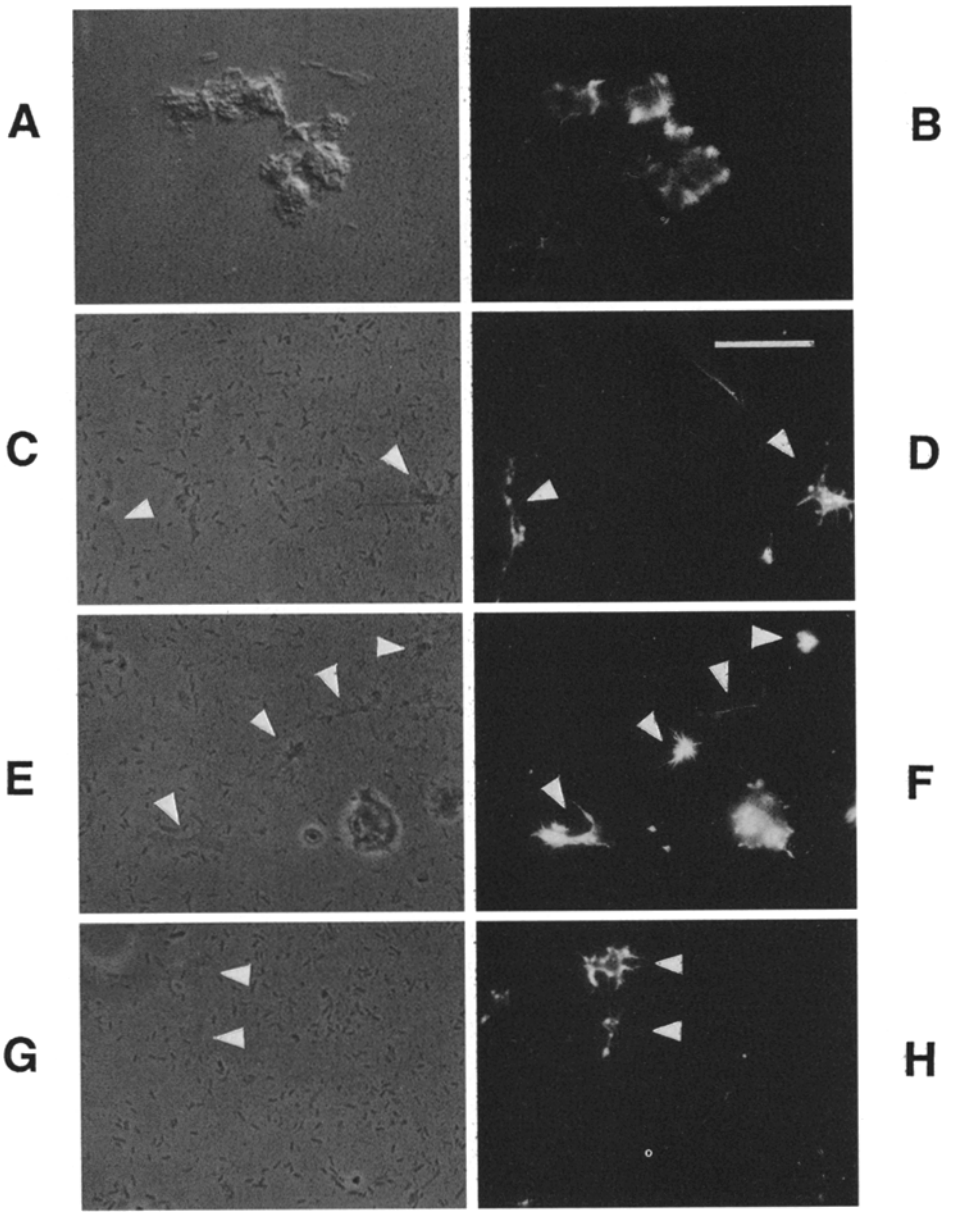

Figure 12. Fragments of cytoplasm are lost during locomotion of 34 kD$^-$/hyg cells on a glass substrate. AX2 (*A* and *B*) and 34 kD$^-$/hyg (*C–H*) cells were allowed to migrate on a glass slide, followed by fixation and staining with rhodamine phalloidin. Bits of cytoplasm lost from the 34-kD$^-$/hyg cells during locomotion and deposited on the substrate are stained with rhodamine-phalloidin (*D*, *F*, and *H*), and are marked with arrowheads in the corresponding light micrographs (*C*, *E*, and *G*). Bar, 20 μm.

could reflect a higher stability of cross-linked actin networks caused by the substitution of partially redundant proteins, such as the other actin cross-linking proteins in *Dictyostelium* (see below). In addition, it is possible that changes in filopodia number and length, or the higher persistence of motility in 34 kD$^-$ cells, could be explained by an impairment in the stabilization of newly formed protrusions. Thus, the differences might result from an impairment of stabilization of newly formed domains rather than from a greater stability of cortical cytoplasm and consequent inhibition of its rearrangement. Additional studies will be required to attempt to unravel such fine mechanistic differences.

A difference in the phenotype of cells lacking the 34-kD protein derived from the strains AX2 and AX3 is not particularly surprising. Clear differences between the parental lines are evident even though they are both derived from NC-4 (Watts and Ashworth, 1970; Loomis, 1971) and bear mutations in the same two loci (axeB and axeC) conferring the axenic phenotype (Williams et al., 1974*a*,*b*). The morphology, speed, and persistence of motility of the AX3 derived line DH1 differs considerably from AX2 (Table I). AX2 and AX3 differ in the requirement for exogenous cAMP pulsing to stimulate competence for aggregation during starvation in shaking suspension (Beug et al., 1973). In addition, strains lacking the gelation factor (ABP 120) derived from AX2 and AX3 differ markedly in phenotype (see above). Finally, and most generally significant, the effects of mutations and/or knockouts are frequently strain dependent in other species, including yeast, *Caenorhabditis elegans*, and the mouse, because of unknown differences in host background, suppressors, synthetic effects, or local effects on expression of nearby genes (see for example Threadgill et al., 1995; Sibilia and Wagner, 1995; Lemmon and Jones, 1987; Sundaram and Han, 1996; Olson et al., 1996). Thus, it is likely that additional differences will likely emerge as data are gathered. The challenge will be to understand the genetic and mechanistic basis of such

differences that should provide significant new insights to the cytoskeletal systems under investigation.

A number of explanations might account for a mild phenotype of a gene knockout of an abundant and highly conserved protein, including (*a*) a modest or specialized function not tested under the laboratory conditions used; (*b*) compensatory changes in other cellular components; (*c*) redundancy or partial redundancy (Brookfield, 1992; Thomas, 1993); and (*d*) superfluous expression (Erickson, 1993). An insignificant or specialized function seems unlikely, since the protein accumulates in a variety of dynamic cytoskeletal structures and is highly conserved from invertebrates to mammals (Furukawa and Fechheimer, 1990; Johns et al., 1988). Compensation was not detected in two other major actin cross-linking proteins, α-actinin and the gelation factor (ABP 120), although changes in the abundance of the other actin cross-linking proteins cannot be ruled out. Superfluous expression seems unlikely for a highly conserved protein that is expressed at high levels in a single-celled invertebrate organism. Thus, we invoke a model of functional redundancy to explain our observations.

The limited range and severity of phenotypes observed is explained by proposing that other actin cross-linking proteins were able to largely subsume the role(s) in the absence of the 34-kD protein. Some aspect of the function(s) is not appropriately performed and/or regulated when the redundant proteins assume the role, resulting in the observed aspects of the phenotype. A central feature of the model is that the phenotype(s) of the single mutants do not reflect all processes to which the protein contributes in wild-type cells, but only the aspects of the function that cannot be fully compensated by substitution of a redundant protein. Since some altered phenotypes are observed in the 34-kD$^-$ cells, the redundancy is partial.

This model of functional redundancy includes simple substitution, changes in localization, and/or changes in level of expression of the various actin cross-linking proteins in response to the absence of the 34-kD protein. The failure of redundant proteins to perform identically to the 34-kD protein could potentially be explained by a variety of differences among the polypeptides. First, the failure could be caused by a difference in regulation of the actin cross-linking activity. The actin cross-linking activity of the 34-kD protein is inhibited in the presence of micromolar calcium (Fechheimer and Taylor, 1984; Fechheimer, 1987), while the activity of some other actin cross-linking proteins, such as elongation factor 1α (Demma et al., 1990), p30b (Brown, 1985), and gelation factor (Condeelis et al., 1981), are calcium insensitive in their interactions with actin. The substitution of a calcium-insensitive actin cross-linking protein for a calcium-sensitive activity could result in actin networks that are less readily rearranged. It is noteworthy in this regard that neutrophils migrating in a three-dimensional matrix fail to retract their tails and shed blebs of cytoplasm if their free calcium concentration is buffered to prevent any calcium transients (Mandeville et al., 1997). Yet, it is possible that other differences between the actin cross-linking proteins are most central, and calcium regulation is not the key. Experimental support for this possibility comes from the observation that a calcium-insensitive version of *Dictyostelium* α-actinin is still capable of rescuing the defect in cells lacking both α-actinin and the gelation factor (Witke et al., 1993).

Alternate differences in the manner of regulation of the actin cross-linking proteins that could explain the results are also consistent with existing data. Changes in intracellular pH can regulate the actin cross-linking of α-actinin (Fechheimer et al., 1982) and of eEF1-α (Edmonds et al., 1995), while the actin cross-linking activity of the 34-kD protein is pH insensitive (Fechheimer and Taylor, 1984). The 34-kD protein and eEF1-α are both present in filopodia in wild-type cells (Fechheimer, 1987; Demma et al., 1990; Okazaki and Yumura, 1995), so the suggestion that the mode of regulation of filopodial dynamics might change in the absence of the 34-kD protein is quite plausible.

Differences in the affinity, kinetics, and geometry of binding of the cross-linking proteins could also account for the failure of other cross-linking proteins to substitute for the 34-kD protein. For example, it has been proposed that some actin-binding proteins direct the formation of isotropic gels, while others favor the formation of bundles of filaments (Matsudaira, 1991; Otto, 1994). The affinity of an actin cross-linking protein for actin is also related to the ability to rearrange networks, and can influence the formation of gelled or bundled cross-linked actin networks (Wachsstock et al., 1993, 1994). In addition, the rheological properties of actin and of cross-linked actin networks are extremely sensitive to the time scale on which the measurement is made (Sato et al., 1987), and rheological studies of networks of actin with either α-actinin or the gelation factor reveal significantly different dynamic mechanical properties (Janssen et al., 1996). Thus, differences in the kinetics of rearrangement of cross-links formed by different cross-linking proteins could dramatically affect the behavior of the cytoplasm in cells lacking one or more of the actin cross-linking proteins.

Finally, differences in the abundance, localization, membrane association, or interaction with other cellular structures could also account for the failure of other cross-linking proteins to substitute for the 34-kD protein. The distributions of the actin cross-linking proteins are under close spatial regulation, since double labeling experiments have demonstrated that α-actinin, the 34-kD protein, gelation factor (ABP 120), elongation factor 1α, and p30b all have distinct localizations in *Dictyostelium* amoebae (Okazaki and Yumura, 1995; Furukawa and Fechheimer, 1994; Fechheimer et al., 1994; Carboni and Condeelis, 1985). In addition, comitin (Weiner et al., 1993), the putative spectrin homologue (Bennett and Condeelis, 1988), and the 34-kD protein (Fechheimer et al., 1994) can be membrane associated at least under some conditions, while the other cross-linking proteins appear to lack such interactions. All of these factors suggest differences in the function of the proteins in wild-type cells, and could account for the inability to compensate completely for the absence of the 34-kD protein.

Specific elimination of actin cross-linking proteins has also been performed in other species. For example, a deficiency of fimbrin causes abnormal morphogenesis in yeast (Adams et al., 1991). Lack of actin-binding protein (filamin) in tumor cells results in deficient locomotion, surface blebbing, and ion channel activation linked to cell volume regulation (Cunningham et al., 1992; Cantiello et al.,

1993). Mutations in *Drosophila* fascin (singed) results in defects in cytoplasmic streaming during oogenesis and abnormal bristle morphogenesis associated with defects in the formation of actin bundles (Cant et al., 1994; Cant and Cooley, 1996). The presence of multiple actin-binding proteins in these systems suggests that redundancy may also occur, and that the observed phenotypes are indicative of the subset of the functions that cannot be suitably compensated. The numerous examples of synthetic phenotypes resulting from mutations of combinations of proteins in the yeast system (Welch et al., 1994) and limited examples in the *Dictyostelium* system (Ostap and Pollard, 1996) indicate that this avenue of investigation is likely to be instructive. Future studies of cells bearing mutations in combinations of actin cross-linking proteins in *Dictyostelium* will allow us to test this proposal, and to unravel their shared and unique contributions to the processes of cell movement and morphogenesis.

We thank Dr. Karl Saxe for the PCR method using whole lysed *Dictyostelium* amoebae as template. We thank Dr. Jacob Franke for supplying plasmid pJB1 and DH1 cells, and Dr. Tom Egelhoff for pDE109. The facilities of the University of Georgia Center for Advanced Ultrastructural Research were used for quantitative measurements of filopodia. M. Fechheimer acknowledges the assistance of Anna Robbins and Chris Hammel in genomic cloning/construction of the gene replacement vectors, and for transformation of DH1, respectively.

This work was supported by National Science Foundation Molecular and Cellular Biosciences 9405738, Deutsche Forschungsgemeinschaft No. 113/5-4, and European Union grant CHRX-CT93-0250.

Received for publication 15 April 1996 and in revised form 22 August 1996.

References

Adams, A.E.M., D. Botstein, and D.G. Drubin. 1991. Requirement of yeast fimbrin for actin organization and morphogenesis in vivo. *Nature (Lond.).* 354:404–408.

Bennett, H., and J. Condeelis. 1988. Isolation of an immunoreactive analog of brain fodrin that is associated with the cell cortex of *Dictyostelium* amoebae. *Cell Motil. Cytoskel.* 11:303–317.

Beug, H., F.E. Katz, and G. Gerisch. 1973. Dynamics of antigenic membrane sites relating to cell aggregation in *Dictyostelium discoideum. J. Cell Biol.* 56: 647–658.

Brink, M., G. Gerisch, G. Isenberg, A.A. Noegel, J.E. Segall, E. Wallraff, and M. Schleicher. 1990. A *Dictyostelium* mutant lacking an F-actin cross-linking protein, the 120-kD gelation factor. *J. Cell Biol.* 111:1477–1489.

Brookfield, J. 1992. Can genes be truly redundant? *Curr. Biol.* 2:553–554.

Brown, S.S. 1985. A calcium insensitive actin-crosslinking protein from *Dictyostelium discoideum. Cell Motil.* 5:529–543.

Cant, K., and L. Cooley. 1996. Single amino acid mutations in *Drosophila* fascin disrupt actin bundling function in vivo. *Genetics.* 143:249–258.

Cant, K., B.A. Knowles, M.S. Mooseker, and L. Cooley. 1994. *Drosophila* singed, a fascin homolog, is required for actin bundle formation during oogenesis and bristle extension. *J. Cell Biol.* 125:369–380.

Cantiello, H.F., A.G. Prat, J.U. Bonventre, C.C. Cunningham, J.H. Hartwig, and D.A. Ausiello. 1993. Actin-binding protein contributes to cell-volume regulatory ion channel activation in melanoma cells. *J. Biol. Chem.* 268: 4596–4599.

Carboni, J.M., and J.S. Condeelis. 1985. Ligand-induced changes in the location of actin, myosin, 95 k (α-actinin), and 120 k protein in amoebae of *Dictyostelium discoideum. J. Cell Biol.* 100:1894–1993.

Caterina, M.J., J.L.S. Milne, and P.N. Devreotes. 1994. Mutation of the third intracellular loop of the cAMP receptor, cAR1, of *Dictyostelium* yields mutants impaired in multiple signaling pathways. *J. Biol. Chem.* 269:1523–1532.

Claviez, M., K. Pagh, H. Maruta, W. Baltes, P. Fisher, and G. Gerisch. 1982. Electron microscopic mapping of monoclonal antibodies on the tail region of *Dictyostelium* myosin. *EMBO (Eur. Mol. Biol. Organ.) J.* 1:1017–1022.

Condeelis, J., J. Salisbury, and K. Fujiwara. 1981. A new protein that gels F-actin in the cell cortex of *Dictyostelium discoideum. Nature (Lond.).* 292:161–163.

Condeelis, J.S., and M. Vahey. 1982. A calcium and pH-regulated protein from *Dictyostelium discoideum* that cross-links filaments. *J. Cell Biol.* 94:466–471.

Cox, D., J. Condeelis, D. Wessels, D. Soll, H. Kern, and D.A. Knecht. 1992. Targeted disruption of the ABP-120 gene leads to cells with altered motility. *J. Cell Biol.* 116:943–955.

Cox, D., J.A. Ridsdale, J. Condeelis, and J. Hartwig. 1995. Genetic deletion of ABP-120 alters the three dimensional organization of actin filaments in *Dictyostelium* pseudopods. *J. Cell Biol.* 128:819–835.

Cox, D., D. Wessels, D.R. Soll, J. Hartwig, and J. Condeelis. 1996. Re-expression of ABP-120 rescues cytoskeletal, motility, and phagocytosis defects of ABP-120$^-$ *Dictyostelium* mutants. *Mol. Biol. Cell.* 7:803–823.

Cunningham, C.C., J.B. Gorlin, D.J. Kwiatkowski, J.H. Hartwig, P.A. Janmey, H.R. Byers, and T.P. Stossel. 1992. Actin-binding protein requirement for cortical stability and efficient locomotion. *Science (Wash. DC).* 255:325–327.

de Hostos, E.L., C. Rehfuess, B. Bradtke, D.R. Waddell, R. Albrecht, J. Murphy, and G. Gerisch. 1993. *Dictyostelium* mutants lacking the cytoskeletal protein coronin are defective in cytokinesis and cell motility. *J. Cell Biol.* 120:163–173.

De Lozanne, A., and J.A. Spudich. 1987. Disruption of the *Dictyostelium* myosin heavy chain gene by homologous recombination. *Science (Wash. DC).* 236:1086–1091.

Demma, M., V. Warren, R. Hock, S. Dharmawardhane, and J. Condeelis. 1990. Isolation of an abundant 50,000 dalton actin filament bundling protein from *Dictyostelium discoideum. J. Biol. Chem.* 265:2286–2291.

Early, A.E., J.G. Williams, H.E. Meyer, S.B. Por, E. Smith, K.L. Williams, and A.A. Gooley. 1988. Structural characterization of *Dictyostelium discoideum* prespore-specific gene D19 and of its product, cell surface glycoprotein PsA. *Mol. Cell. Biol.* 8:3458–3466.

Edmonds, B.T., J. Murray, and J. Condeelis. 1995. pH regulation of the F-actin binding properties of *Dictyostelium* elongation factor 1-α. *J. Biol. Chem.* 270: 15222–15230.

Egelhoff, T.T., S.S. Brown, D.J. Manstein, and J.A. Spudich. 1989. Hygromycin resistance as a selectable marker in *Dictyostelium discoideum. Mol. Cell. Biol.* 9:1965–1968.

Erickson, H.P. 1993. Gene knockouts of c-src, transforming growth factor β1, and tenascin suggest superfluous, nonfunctional expression of proteins. *J. Cell Biol.* 120:1079–1081.

Faix, J., G. Gerisch, and A.A. Noegel. 1992. Overexpression of the csA cell adhesion molecule under its own cAMP-regulated promoter impairs morphogenesis in *Dictyostelium discoideum. J. Cell Sci.* 102:203–214.

Fechheimer, M., J. Brier, M. Rockwell, E.J. Luna, and D.L. Taylor. 1982. A calcium and pH regulated actin binding protein from *D. discoideum. Cell Motil.* 2:287–308.

Fechheimer, M., and D.L. Taylor. 1984. Isolation and characterization of a 30,000-dalton calcium-sensitive actin crosslinking protein from *Dictyostelium discoideum. J. Biol. Chem.* 259:4514–4520.

Fechheimer, M. 1987. The *Dictyostelium discoideum* 30,000 dalton calcium-sensitive actin-bundling protein is selectively present in filopodia. *J. Cell Biol.* 104:1539–1551.

Fechheimer, M., D. Murdock, M. Carney, and C.V.C. Glover. 1991. Isolation and sequencing of cDNA clones encoding the *Dictyostelium discoideum* 30,000 dalton actin-bundling protein. *J. Biol. Chem.* 266:2883–2889.

Fechheimer, M. 1993. Small (M_r <35,000) actin crosslinking proteins. *In* Guidebook to the Cytoskeletal and Motor Proteins. T.E. Kreis and R.D. Vale, editors. Oxford University Press, Oxford, U.K. 74–76.

Fechheimer, M., H.M. Ingalls, R. Furukawa, and E.J. Luna. 1994. Association of the *Dictyostelium* 30,000 dalton actin bundling protein with contact regions. *J. Cell Sci.* 107:2393–2401.

Feinberg, A.P., and B. Vogelstein. 1983. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Anal. Biochem.* 132:6–13.

Fisher, P.R., R. Merkl, and G. Gerisch. 1989. Quantitative analysis of cell motility and chemotaxis in *Dictyostelium discoideum* by using an image processing system and a novel chemotaxis chamber providing stationary chemical gradients. *J. Cell Biol.* 108:973–984.

Franke, J., and R. Kessin. 1977. A defined minimal medium for axenic strains of *Dictyostelium discoideum. Proc. Natl. Acad. Sci. USA.* 74:2157–2161.

Furukawa, R., and M. Fechheimer. 1990. Localization, expression, evolutionary conservation, and structure of the 30,000 dalton actin bundling protein of *Dictyostelium discoideum. Dev. Genet.* 11:362–368.

Furukawa, R., S. Butz, E. Fleischmann, and M. Fechheimer. 1992. The *Dictyostelium discoideum* 30,000 dalton protein contributes to phagocytosis. *Protoplasma.* 169:18–27.

Furukawa, R., and M. Fechheimer. 1994. Differential localization of α-actinin and the 30 kD actin-bundling protein in the cleavage furrow, phagocytic cup, and contractile vacuole of *Dictyostelium discoideum. Cell Motil. Cytoskel.* 29:46–56.

Gibbons, J.D. 1985. Nonparametric Methods for Quantitative Analysis. 2nd ed. American Sciences Press, Inc., Columbus, OH. 481 pp.

Haugwitz, M., A.A. Noegel, J. Karakesisoglou, and M. Schleicher. 1994. *Dictyostelium* amebas that lack G-actin sequestering profilins show defects in F-actin content, cytokinesis, and development. *Cell.* 79:303–314.

Hock, R.S., and J.S. Condeelis. 1987. Isolation of 240-kilodalton actin-binding protein from *Dictyostelium discoideum. J. Biol. Chem.* 262:394–400.

Jacquet, M., R. Guilbaud, and H. Garreau. 1988. Sequence analysis of the DdPyr5-6 gene coding for UMP synthase in *Dictyostelium discoideum* and comparison with orotate phosphoribosyl transferases and OMP decarboxylases. *Mol. Gen. Genet.* 211:441–445.

Janson, L.W., J. Kolega, and D.L. Taylor. 1991. Modulation of contraction by

gelation/solation in a reconstituted motile model. *J. Cell Biol.* 114:1005–1015.

Janssen, K.-P., L. Eichinger, P.A. Janmey, A.A. Noegel, M. Schliwa, W. Witke, and M. Schleicher. 1996. Viscoelastic properties of F-actin solutions in the presence of normal and mutated actin-binding proteins. *Arch. Biochem. Biophys.* 325:183–189.

Jermyn, K.A., M. Berks, R.R. Kay, and J.E. Williams. 1987. Two distinct classes of prestalk-enriched mRNA sequences in *Dictyostelium discoideum*. *Development (Camb.).* 100:745–755.

Johns, J.A., A.M. Brock, and J.D. Pardee. 1988. Colocalization of F-actin and 34-kilodalton actin bundling protein in *Dictyostelium* and cultured fibroblasts. *Cell Motil. Cytoskel.* 9:205–218.

Klein, P.S., T.J. Sun, C.L. Saxe III, A.R. Kimmel, R.L. Johnson, and P.N. Devreotes. 1988. A chemoattractant receptor controls development in *Dictyostelium discoideum*. *Science (Wash. DC).* 241:1467–1472.

Knecht, D.A., S.M. Cohen, W.F. Loomis, and H.F. Lodish. 1986. Developmental regulation of *Dictyostelium discoideum* actin gene fusions carried on low-copy and high-copy transformation vectors. *Mol. Cell. Biol.* 6:3973–3983.

Knecht, D.A., and W.F. Loomis. 1987. Antisense RNA inactivation of myosin heavy chain gene expression in *Dictyostelium discoideum*. *Science (Wash. DC).* 241:1081–1086.

Laemmli, U.K. 1970. Cleavage of structural proteins during assembly of the head of bacteriophage T4. *Nature (Lond.).* 227:680–685.

Lemmon, S.K., and E.W. Jones. 1987. Clathrin requirement for normal growth of yeast. *Science (Wash. DC).* 238:504–509.

Loomis, W.F. 1971. Sensitivity of *Dictyostelium discoideum* to nucleic acid analogues. *Exp. Cell Res.* 64:484–486.

Mandeville, J.T.H., M.A. Lawson, and F.R. Maxfield. 1997. Dynamic imaging of neutrophil migration in three dimensions: mechanical interactions between cells and matrix. *J. Leukocyte Biol.* In press.

Mann, S.K.O., P.N. Devreotes, S. Eliott, K. Jermyn, A. Kuspa, M. Fechheimer, R. Furukawa, C.A. Parent, J. Segall, G. Shaulsky, et al. 1994. Cell biological, molecular genetic, and biochemical methods to examine *Dictyostelium*. *In* Cell Biology: A Laboratory Handbook. Vol. 1. J.E. Celis, editor. Academic Press, San Diego, CA. 412–451.

Matsudaira, P. 1991. Modular organization of actin crosslinking proteins. *Trends Biochem. Sci.* 16:87–92.

Newell, P.C., A. Telser, and M. Sussmann. 1969. Alternative developmental pathways determined by environmental conditions in the cellular slime mold *Dictyostelium discoideum*. *J. Bacteriol.* 100:763–768.

Noegel, A.A., B.A. Metz, and K.L. Williams. 1985. Developmentally regulated transcription of *Dictyostelium discoideum* plasmid Ddp1. *EMBO (Eur. Mol. Biol. Organ.) J.* 4:3797–3803.

Noegel, A., W. Witke, and M. Schleicher. 1987. Calcium-sensitive non-muscle α-actinin contains EF-hand structures and highly conserved regions. *FEBS Lett.* 221:391–396.

Noegel, A., S. Rapp, F. Lottspeich, M. Schleicher, and M. Stewart. 1989. The *Dictyostelium* gelation factor shares a putative actin-binding site with α-actinin and dystrophin and also has a rod domain containing six 100 residue motifs that appear to have a cross-β conformation. *J. Cell Biol.* 108:607–618.

Okazaki, K., and S. Yumura. 1995. Differential association of three actin-bundling proteins with microfilaments in *Dictyostelium* amebas. *Eur. J. Cell Biol.* 66:75–81.

Olson, E.N., H.-H. Arnold, P.W.J. Rigby, and B.J. Wold. 1996. Know your neighbors: three phenotypes in null mutants of the myogenic bHLH gene MRF4. *Cell.* 85:1–4.

Ostap, E.M., and T.D. Pollard. 1996. Overlapping functions of myosin-I isoforms? *J. Cell Biol.* 133:221–224.

Otto, J.J. 1994. Actin-bundling proteins. *Curr. Opin. Cell Biol.* 6:105–109.

Rivero, F., B. Köppel, B. Peracino, S. Bozzaro, F. Siegert, C.J. Weijer, M. Schleicher, R. Albrecht, and A.A. Noegel. 1996. The role of the cortical cytoskeleton: F-actin cross-linking proteins protect against osmotic stress, ensure cell size, cell shape and motility, and contribute to phagocytosis and development. *J. Cell Sci.* In press.

Sambrook, J., E.F. Fritsch, and T. Maniatis. 1989. Molecular Cloning. A Laboratory Manual. 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Sato, M., W.H. Schwartz, and T.D. Pollard. 1987. Dependence of the mechanical properties of actin/alpha-actinin gels on deformation rate. *Nature (Lond.).* 325:828–830.

Schindl, M., E. Wallraff, B. Deubzer, W. Witke, G. Gerisch, and E. Sackmann. 1995. Cell-substrate interactions and locomotion of *Dictyostelium* wild-type and mutants defective in three cytoskeletal proteins: a study using quantitative reflection interference contrast microscopy. *Biophys. J.* 68:1177–1190.

Schleicher, M., and A.A. Noegel. 1992. Dynamics of the *Dictyostelium* cytoskeleton during chemotaxis. *The New Biologist.* 4:461–472.

Schleicher, M., A.A. Noegel, T. Schwarz, E. Wallraff, M. Brink, J. Faix, G. Gerisch, and G. Isenberg. 1988. A *Dictyostelium* mutant with severe defects in α-actinin: its characterization using cDNA probes and monoclonal antibodies. *J. Cell Sci.* 90:59–71.

Segall, J.E., P.R. Fisher, and G. Gerisch. 1987. Selection of chemotaxis mutants of *Dictyostelium discoideum*. *J. Cell Biol.* 104:151–161.

Sibilia, M., and E.F. Wagner. 1995. Strain-dependent epithelial defects in mice lacking the EGF receptor. *Science (Wash. DC).* 269:234–238.

Smith, P.K., R.I. Krohn, G.T. Hermanson, A.K. Mallia, F.H. Gartner, M.D. Provenzano, E.K. Fujimoto, N.M. Goeke, B.J. Olson, and D.C. Klenk. 1985. Measurement of protein using bicinchoninic acid. *Anal. Biochem.* 150:76–85.

St.-Pierre, B., C. Couture, A. Larouche, and D. Pallotta. 1993. Two developmentally regulated mRNAs encoding actin-binding proteins in *Physarum polycephalum*. *Biochim. Biophys. Acta.* 1173:107–110.

Sundaram, M., and M. Han. 1996. Control and integration of cell signaling pathways during *C. elegans* vulval development. *BioEssays.* 18:473–480.

Swanson, J.A., B.D. Yirinec, and S.C. Silverstein. 1985. Phorbol esters and horseradish peroxidase stimulate pinocytosis and redirect the flow of pinocytosed fluid in macrophages. *J. Cell Biol.* 100:851–859.

Taylor, D.L., and J.S. Condeelis. 1979. Cytoplasmic structure and contractility in amoeboid cells. *Int. Rev. Cytol.* 56:57–144.

Taylor, D.L., and M. Fechheimer. 1982. Cytoplasmic structure and contractility: the solation-contraction coupling hypothesis. *Philos. Trans. R. Soc. Lond. B. Biol. Sci.* 299:185–197.

Thomas, J.H. 1993. Thinking about genetic redundancy. *Trends Genet.* 9:395–399.

Threadgill, D.W., A.A. Dlugosz, L.A. Hansen, T. Tennenbaum, U. Lichti, D. Yee, C. LaMantia, T. Mourton, K. Herrup, R.C. Harris, et al. 1995. Targeted disruption of mouse EGF receptor: effect of genetic background on mutant phenotype. *Science (Wash. DC).* 269:230–234.

Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA.* 76:4350–4354.

Wachsstock, D.H., W.H. Schwarz, and T.D. Pollard. 1993. Affinity of α-actinin for actin determines the structure and mechanical properties of actin filament gels. *Biophys. J.* 65:205–214.

Wachsstock, D.H., W.H. Schwarz, and T.D. Pollard. 1994. Cross-linker dynamics determine the mechanical properties of actin gels. *Biophys. J.* 66:801–809.

Wallraff, E., M. Schleicher, M. Modersitzki, D. Rieger, G. Isenberg, and G. Gerisch. 1986. Selection of *Dictyostelium* mutants defective in cytoskeletal proteins: the use of an antibody that binds to the ends of α-actinin rods. *EMBO (Eur. Mol. Biol. Organ.) J.* 5:61–67.

Watts, D.J., and J.M. Ashworth. 1970. Growth of myxamoebae of the cellular slime mould *Dictyostelium discoideum* in axenic culture. *Biochem. J.* 119: 171–174.

Weber, I., E. Wallraff, R. Albrecht, and G. Gerisch. 1995. Motility and substratum adhesion of *Dictyostelium* wild-type and cytoskeletal mutant cells: a study by RICM/bright-field double-view image analysis. *J. Cell Sci.* 108: 1519–1530.

Weiner, O.H., J. Murphy, G. Griffiths, M. Schleicher, and A.A. Noegel. 1993. The actin-binding protein comitin (p24) is a component of the Golgi apparatus. *J. Cell Biol.* 123:23–34.

Welch, M.D., D.A. Holtzman, and D.G. Drubin. 1994. The yeast actin cytoskeleton. *Curr. Opin. Cell Biol.* 6:110–119.

Williams, K.L., R.H. Kessin, and P.C. Newell. 1974*a*. Genetics of growth in axenic medium of the cellular slime mould *Dictyostelium discoideum*. *Nature (Lond.).* 247:142–143.

Williams, K.L., R.H. Kessin, and P.C. Newell. 1974*b*. Parasexual genetics in *Dictyostelium discoideum*: mitotic analysis of acriflavin resistance and growth in axenic medium. *J. Gen. Microbiol.* 84:59–69.

Williams, K.L., and P.C. Newell. 1976. A genetic study in the cellular slime mold *Dictyostelium discoideum* using complementation analysis. *Genetics.* 82:287–307.

Witke, W., W. Nellen, and A.A. Noegel. 1987. Homologous recombination in the *Dictyostelium* α-actinin gene leads to an altered mRNA and lack of protein. *EMBO (Eur. Mol. Biol. Organ.) J.* 6:4143–4148.

Witke, W., M. Schleicher, and A.A. Noegel. 1992. Redundancy in the microfilament system: abnormal development of *Dictyostelium* cells lacking two F-actin cross-linking proteins. *Cell.* 68:53–62.

Witke, W., A. Hofmann, B. Koppel, M. Schleicher, and A.A. Noegel. 1993. The Ca^{2+}-binding domains in nonmuscle type α-actinin: biochemical and genetic analysis. *J. Cell Biol.* 121:599–606.

Zigmond, S.H., R. Furukawa, and M. Fechheimer. 1992. Inhibition of actin filament depolymerization by the *Dictyostelium* 30,000-D actin-bundling protein. *J. Cell Biol.* 119:559–567.