A *Dictyostelium* Mutant Lacking an F-Actin Cross-Linking Protein, the 120-kD Gelation Factor

Marijke Brink, Günther Gerisch, Gerhard Isenberg, Angelika A. Noegel, Jeffrey E. Segall, Eva Wallraff, and Michael Schleicher

Max-Planck-Institut für Biochemie, 8033 Martinsried, Federal Republic of Germany

Abstract. Actin-binding proteins are known to regulate in vitro the assembly of actin into supramolecular structures, but evidence for their activities in living nonmuscle cells is scarce. Amebae of *Dictyostelium discoideum* are nonmuscle cells in which mutants defective in several actin-binding proteins have been described. Here we characterize a mutant deficient in the 120-kD gelation factor, one of the most abundant F-actin cross-linking proteins of *D. discoideum* cells. No F-actin cross-linking activity attributable to the 120-kD protein was detected in mutant cell extracts, and antibodies recognizing different epitopes on the polypeptide showed the entire protein was lacking. Under the conditions used, elimination of the gelation factor did not substantially alter growth, shape, motility, or chemotactic orientation of the cells towards a cAMP source. Aggregates of the mutant developed into fruiting bodies consisting of normally differentiated spores and stalk cells. In cytoskeleton preparations a dense network of actin filaments as typical of the cell cortex, and bundles as they extend along the axis of filopods, were recognized. A significant alteration found was an enhanced accumulation of actin in cytoskeletons of the mutant when cells were stimulated with cyclic AMP. Our results indicate that control of cell shape and motility does not require the fine-tuned interactions of all proteins that have been identified as actin-binding proteins by in vitro assays.

SHAPE changes in motile nonmuscle cells are accompanied by rearrangements in the microfilament system. A variety of proteins has been isolated from such cells that regulate the in vitro polymerization of actin, the cross-linking of actin filaments into a network, or bundling of these filaments (reviewed by Stossel et al., 1985; Pollard and Cooper, 1986). The in vivo role of these actin-binding proteins has been inferred from their colocalization with microfilament assemblies in permeabilized cells or isolated cell cortices (Condeelis et al., 1981, 1988; Ogihara et al., 1988), and also from changes in cells induced by microinjection of these proteins or specific antibodies (Füchtbauer et al., 1983; Cooper et al., 1987; Höner and Jockusch, 1988).

A powerful tool for studying the function of a protein in living cells is its elimination by mutation. This has been done only for a small number of cytoskeletal proteins. *Dictyostelium discoideum* is a eukaryotic microorganism in which genetic studies on the control of cytokinesis, endocytosis, motility and chemotaxis of nonmuscle cells are possible. Cells of *D. discoideum* display ameboid movement like many other nonmuscle cells, and contain actin-binding proteins

that share portions of their sequences with cytoskeletal proteins in vertebrates (Schleicher et al., 1988*b*). For instance, an NH₂-terminal region of *D. discoideum* α-actinin (Noegel et al., 1987) is highly homologous to that of chicken fibroblast α-actinin (Baron et al., 1987); gelsolin, the F-actin capping and severing protein from higher eukaryotes (Kwiatkowski et al., 1986), is essentially a duplicated version of severin, the major F-actin fragmenting protein from *D. discoideum* (André et al., 1988). Because *D. discoideum* cells are haploid, recessive mutations affecting the expression of specific proteins are easy to detect by the use of antibodies (Wallraff et al., 1986; André et al., 1989). In addition to chemical mutagenesis, gene inactivation by transformation with cloned cDNA fragments in antisense orientation or homologous recombination has been applied to eliminate specific cytoskeletal proteins in *D. discoideum* cells (Knecht and Loomis, 1987; De Lozanne and Spudich, 1987; Witke et al., 1987).

Genetic analyses of the cytoskeletal system are complicated by the fact that the proteins constituting this system interact with each other in a complicated manner. They form a network in which certain functions appear to be guaranteed by more than a single protein. Therefore, if a protein is eliminated, the following possibilities have to be considered. (*a*) The function of the protein is vital and the protein is the only one serving this function. A mutation resulting in loss of the protein would be lethal. (*b*) The function of the protein is vi-

tal but is guaranteed by a family of proteins. In this case the result depends on the specific functional design of the cytoskeletal system. If cell shape and motility would depend on the fine-tuned activity of all the proteins that interact with actin in vitro, elimination of one of these proteins should substantially alter the behavior of the cells. If, however, some of these proteins are replaceable by other proteins, then lack of the protein will have only minor and possibly undetectable consequences. Perhaps a protein being replaceable under normal circumstances is required under certain stress conditions. An intermediate result would be predicted if proteins have overlapping but nonidentical functions (Bray and Vasiliev, 1989). (c) An extreme case would be a protein that has no in vivo function corresponding to its in vitro activity.

In previous papers a mutant deficient in α-actinin was studied, which is the most abundant actin-crosslinking protein of D. discoideum cells (Wallraff et al., 1986; Schleicher et al., 1988a). The finding that no substantial changes in cell behavior were observed in the mutant raised the question of whether other F-actin cross-linking proteins are required for maintaining normal cell functions. The cross-linking protein next to α-actinin in terms of its in vitro activity is an F-actin gelation factor with an apparent subunit molecular mass of 120 kD as determined by SDS-PAGE (Condeelis et al., 1981). Like α-actinin, the 120-kD factor is a rod-shaped molecule composed of two identical filamentous subunits that form actin-binding sites at each of the two ends of the rod (Condeelis et al., 1984). The cDNA-derived amino acid sequence suggests that an actin-binding domain similar to that of α-actinin is located at the NH_2 terminus and that the rod is formed by six repeats with cross-beta conformation (Noegel et al., 1989b). The 120-kD gelation factor differs from α-actinin in not being inhibited by Ca^{2+} (Condeelis et al., 1984) and also in its effect on the actin-stimulated Mg^{2+}-ATPase of myosin. While the 120-kD gelation factor inhibits, α-actinin stimulates the ATPase (Condeelis et al., 1984).

In cells stimulated with the chemoattractant cAMP, the 120-kD gelation factor was found to be enriched in newly formed pseudopods (Condeelis et al., 1988). This finding, together with localization of the 120-kD protein to dense networks of actin filaments and surface projections in isolated cell cortices (Ogihara et al., 1988), suggested a function of this protein in controlling cell motility and determining the shape of the cytoskeleton. In accord with these results, high-voltage EM of gels produced by actin filaments and purified 120-kD gelation factor, revealed a structure reminiscent of the cortical network in D. discoideum cells (Wolosewick and Condeelis, 1986). To study the importance of this protein in vivo we are using the genetic approach, investigating growth, shape, and behavior of cells during development in a mutant lacking the 120-kD gelation factor.

Materials and Methods

Cell Culture and Mutagenesis

AX2-214 was used as wild-type strain. The mutant strain HG1264 was derived from this strain after mutagenesis of growth-phase cells by incubation with 1 mg/ml of 1-methyl-3-nitro-1-nitrosoguanidine (MNNG)[1] in non-

1. Abbreviation used in this paper: MNNG, 1-methyl-3-nitro-1-nitrosoguanidine.

nutrient buffer. The survival of mutagenized cells was ~20%. Mutant strain HG1264 was obtained by screening colony blots with iodinated mAbs (Wallraff et al., 1986).

Normally, wild-type and mutant cells were cultivated axenically at 23°C in nutrient medium with 1.8% maltose according to Watts and Ashworth (1970) up to a density of not more than 5×10^6 cells per ml. Exponentially growing cells were washed twice in cold 17 mM Soerensen phosphate buffer, pH 6.0, resuspended at a density of 1×10^7 cells per ml, and either used directly or starved for 6 or 8 h on a rotary shaker at 150 rpm to obtain developed cells.

For RNA isolation, cells were grown on SM-agar with Klebsiella aerogenes (Sussman, 1966; Williams and Newell, 1976). For the time-course experiment, the cells were harvested, washed free of bacteria, and allowed to develop at 21°C in the light on Millipore filters (Newell et al., 1969).

Rate of growth in axenic culture was measured by determining cell densities at time intervals of 8-12 h. To determine growth of mutant and wild-type cells on bacteria, cells were cloned so that single cells were inoculated on low-nutrient agar plates (Wallraff et al., 1984) together with Escherichia coli B/2. Colony diameters were measured in two directions at daily intervals after incubation of the plates at 23°C.

Genetic Analysis

For mapping the mutation, the haploid cells of mutant HG1264 and of the temperature-sensitive, cobalt-resistant tester strain HU1628 (Wallraff et al., 1984; Welker and Williams, 1985) were fused to diploids in the presence of $CaCl_2$ (Williams and Newell, 1976). The diploids were selected on SM-agar plates containing 300 μg/ml $CoCl_2$ (Williams, 1978) at 27°C. For linkage group analysis the diploids were haploidized on SM agar containing 2 μg/ml of thiabendazole (Welker and Williams, 1980). The haploid segregants were tested for the presence or absence of linkage group markers as well as for binding of iodinated antibodies against the gelation factor in colony blots.

Antibodies

mAbs against severin (mAb 65), the 34-kD subunit of the capping protein (mAb 442), and α-actinin (mAb 19) were described by Schleicher et al. (1984). The antibodies 33-294-17 (mAb 294) and 41-71-21 (mAb 71) against contact site A were described by Bertholdt et al. (1985). Antibodies against the 120-kD gelation factor designated as mAbs 82-250-2, 82-292-6, 82-382-8, 82-405-4, 82-421-5, 82-447-4, 82-454-12, and 82-471-14 are in this paper referred to as mAb 250, 292, 382, 405, 421, 447, 454 and 471, respectively. For the preparation of mAbs against gelation factor, female BALB/c mice were immunized with gelation factor, purified from AX2 cells according to Condeelis et al. (1982), mixed with Freund's adjuvant essentially as described (Schleicher et al., 1984). Spleen cells were fused with 63Ag8-653 cells. Monoclonal IgG was purified from hybridoma culture supernatants by ammonium sulfate precipitation and protein A-Sepharose chromatography as described by Claviez et al. (1982). Purified antibodies were directly iodinated with ^{125}I using the chloramine T method.

A polyclonal antiserum against the gelation factor was raised in a rabbit by subcutaneous injections of purified gelation factor with Freund's adjuvant. The antibodies were affinity purified essentially as described by Fowler and Bennett (1984) and used together with ^{125}I-goat anti-rabbit IgG for indirect labeling of blots after SDS-PAGE.

Rotary Shadowing

Gelation factor and antibody were diluted in 50 mM NH_4HCO_3, yielding final concentrations of 0.2 mg/ml of each protein, incubated over night on ice, diluted with 50 mM NH_4HCO_3 to 0.04 mg/ml of each protein, mixed with one part of glycerol and sprayed at room temperature onto freshly cleaved mica. After drying in vacuum, samples were rotary shadowed at 5°C with platinum/carbon and at ~80°C with carbon, essentially as described by Claviez et al. (1982). Replicas were photographed in a Jeol JEM-100 CX electron microscope at 80 kV.

Limited Tryptic Digestion of Gelation Factor

500 μg of purified gelation factor were incubated with 12 μg of N-tosyl-L-phenylalanine chloromethyl ketone-treated trypsin in 300 μl of 10 mM Tris-HCl, pH 7.8, 1 mM EGTA, 1 mM DTT, 0.02% NaN_3, 250 mM NaCl at 37°C. The reaction was started by addition of trypsin to the protein solution

which was preincubated at 37°C for 2 min. After 1, 2, 5, 10, and 30 min, aliquots were removed from the reaction mixture and frozen in liquid nitrogen.

Cloning and Expression of Gelation Factor-specific cDNA Fragments in pIMS Vectors

cDNA inserts were isolated from recombined λgt11-phages after digestion with Eco RI and cloned into the pIMS vectors (Simon et al., 1988). The plasmids were transformed into *Escherichia coli* JM83 (ara, Δ(lac pro A, B), rspL, φ 80, lac ZΔM15 (r^+_k, m^+_k) (Yanisch-Perron et al., 1985). Colonies were screened for expression of gelation factor fragments by in situ immunoblotting (Simon et al., 1988) using a mixture of the mAbs described.

Fractionation of Mutant and Wild-Type Proteins

Aggregation-competent wild-type and mutant cells were subjected under identical conditions to the first two steps of gelation factor purification, essentially as described for α-actinin (Schleicher et al., 1988a). Fractions containing the gelation factor were detected by SDS-PAGE followed by Coomassie blue staining and by low shear viscometry (MacLean-Fletcher and Pollard, 1980) done at 15 min of incubation at 25°C. Rabbit skeletal muscle actin used in these assays was prepared according to Spudich and Watt (1971) and gel filtrated on Sephacryl S300.

SDS-Gel Electrophoresis and Immunoblotting

Tryptic cleavage products, total cell proteins, fractions obtained during purification, and proteins of the Triton-insoluble cytoskeleton were separated by SDS-PAGE on minislab gels and transferred onto BA85 nitrocellulose filters (Schleicher & Schuell, Inc., Keene, NH) for 1-2 h at 500 mA. Filters were saturated in Tween-buffer (10 mM Tris/HCl, 0.9% NaCl, and 0.05% Tween 20, pH 7.4) for at least 1 h, incubated with iodinated antibody adjusted to 2 × 10^5 cpm per ml, or with an unconjugated first antibody, followed by a ^{125}I-labeled second antibody adjusted to 1 × 10^5 cpm/ml, and finally subjected to autoradiography on Kodak XAR-5 films.

To prevent degradation of the gelation factor in SDS sample buffer samples were frozen in liquid nitrogen, stored at −30°C, mixed during thawing with threefold concentrated SDS sample buffer just before use, boiled for 2-3 min, and immediately loaded. Minislab gels were run at 180 V.

For quantification of the gelation factor during development, antibody-labeled bands were excised for γ-counting. Background determined in an area from underneath the labeled bands was subtracted.

DNA and RNA Analysis

Chromosomal DNA of *D. discoideum* was isolated from partially purified nuclei. For the preparation of nuclei 5 × 10^9 cells were lysed in 150 ml of NP-40-buffer (10 mM magnesium acetate, 10 mM NaCl, 30 mM Hepes buffer, pH 7.5, 10% sucrose, 2% NP-40). The nuclei were pelleted by a low-speed spin and subsequently lysed in EDTA-Sarcosyl solution (0.2 M EDTA, 2% Sarcosyl; pH adjusted to 8.4 with NaOH) at 60°C. The DNA was further purified by CsCl-ethidium bromide gradient centrifugation as described by Noegel et al. (1985a). For Southern blot analysis *D. discoideum* DNA was digested with various restriction enzymes, separated on 0.7% agarose gels in Tris phosphate buffer, pH 7.8 (Maniatis et al., 1982), transferred to nitrocellulose filters (Schleicher & Schuell, Inc.) and probed with radioactively labeled insert DNA of the complete cDNA-clone cDGF10 (Noegel et al., 1989b).

Total cellular RNA of *D. discoideum* was isolated after lysis with 1% SDS and purified by several phenol-chloroform extractions according to Noegel et al. (1985b). For Northern blot analysis, RNA was separated on a 1.2% agarose gel in the presence of formaldehyde (Maniatis et al., 1982), blotted onto nitrocellulose filters and hybridized with nick-translated probes as described (Noegel et al., 1985b).

Labeling of Cryosections

For immunofluorescence labeling of cryosections, wild-type and mutant cells were fixed at 6 h of starvation in a mixture of 1% formaldehyde and 0.01% glutaraldehyde in Soerensen phosphate buffer, pH 6.0, for 15 min at room temperature, followed by 1 h on ice. Fixed cells were pelleted in gelatin, postfixed in 1% formaldehyde for 1 h on ice, and prepared for sectioning according to Tokuyasu (1973). Sections of ∼0.5 μm were obtained on a cryoultramicrotome (FC 4D; Reichert Jung, Vienna) and treated as follows:

5 min with 100 mM glycine in PBS, pH 7.4; twice for 10 min each with 0.5% BSA and 0.1% gelatin in PBS (PBG; Van Bergen en Henegouwen and Leunissen, 1986); 1 h with mAb 382 or mAb 454 (1 μg IgG/ml) diluted in PBG; seven times for 2 min each with PBG; 1 h with a mixture of FITC-labeled goat anti-mouse IgG (Jackson Immunoresearch Laboratories Inc., Avondale, PA; diluted 1:200) and 0.2 μg/ml of TRITC-labeled phalloidin (Sigma Chemical Co.); seven times for 2 min each in PBG. Labeled sections were mounted in semisolid medium (Lennette, 1978) containing 25 mg/ml 1,4-diazobicyclo-(2,2,2)-octane (DABCO; Langanger et al., 1983) and photographed on a Tri-X film (Eastman Kodak Co., Rochester, NY) using a Neofluar 100/1.3 Zeiss phase-contrast objective.

Assays for Capping of Membrane Proteins, Chemotaxis and Cell Motility

To test the mutant cells on their ability to patch and cap membrane proteins they were incubated with 250 μg/ml of mAb 71, an antibody against the protein moiety of contact site A, followed by FITC-labeled goat-anti-mouse IgG as described before (Wallraff et al., 1986; André et al., 1989).

For qualitative evaluation of chemotaxis in the mutant, cells were transferred at 6 h of starvation onto a glass surface and stimulated with micropipettes filled with 1 mM cAMP essentially as described (Gerisch et al., 1975a,b; Gerisch and Keller, 1981; Swanson and Taylor, 1982) and examined in a Zeiss inverted microscope equipped with phase contrast optics (Planapo 100/1.3).

Quantitative data on motility and chemotaxis were obtained using an image-processing system (Segall et al., 1987) and a chamber with which the orientation of wild-type and mutant cells in stable linear gradients of cAMP can be determined (Fisher et al., 1989). The steepness of the gradient was 2.5 × 10^{-8} M cAMP/mm and the average concentration at the site of the cells was 2.5 × 10^{-8} M cAMP.

Determination of Cytoskeleton-associated Actin after Stimulation with cAMP

Cytoskeletons were isolated essentially according to McRobbie and Newell (1983). Cells were harvested at 6 or 8 h of starvation, centrifuged at 100 g for 4 min and resuspended at 5 × 10^7 or 10^8 cells per ml. Samples of 150 μl were dispensed into 1.5-ml Eppendorf microcentrifuge tubes and shaken at room temperature at 1,000 rpm on an IKA Vibrax shaker. Chemoattractant (15 μl cAMP, 10^{-6} M) was then added to each tube and the reaction of the cells was terminated after a preset time of shaking by the addition of 150 μl of a 2× Triton stock solution during shaking (2% Triton X-100, purified for membrane research [Boehringer Mannheim, FRG], 20 mM KCl, 20 mM imidazole, 20 mM EGTA, 4 mM NaN₃, pH 7.0). Subsequently, the tubes were placed on ice for 10-15 min, allowed to warm up to room temperature for 15-30 min with occasional agitation, and spun for 4 min in an Eppendorf centrifuge at 10,000 g. The pellets were resuspended in 300 μl of 2× Triton stock solution diluted 1:1 with 17 mM Soerensen phosphate buffer, pH 6.0. The tubes were recentrifuged, supernatants discarded and the tubes inverted to dry. The protein pellet referred to as the Triton-insoluble-cytoskeleton was analyzed by SDS-PAGE. Gels were stained with 0.1% Coomassie brilliant blue R, destained in methanol/water/acetic acid (2:7:1, by volume), and the stained protein bands were scanned in a CAMAG (Muttenz, Switzerland) electrophoresis scanner with a slit dimension of 0.2 × 3 mm. Changes in actin content were measured by cutting out, weighing, and comparing the appropriate peaks from the scanning traces.

Preparation of Cell Cytoskeletons for EM

Cell cytoskeletons were prepared essentially as described by Claviez et al. (1986). Briefly, cells were allowed to spread for ∼10-20 min on pioloform-coated gold grids (Webster et al., 1978). Then extraction medium, containing 1% Triton X-100 (Boehringer) and 0.05% glutaraldehyde in cytoskeleton stabilizing buffer (20 mM KCl, 2 mM MgSO₄, 10 mM EGTA, 10 mM Pipes, pH 7.0), was added, and the grids were incubated for 5-10 min at room temperature. Samples were washed and postfixed in 2% glutaraldehyde for at least 1 h, the cytoskeletons stained with 2% phosphotungstic acid in bidistilled water adjusted to pH 7.4 with 10% ammonium hydroxide (Pagh et al., 1985), air-dried, and examined in a Jeol JEM-100 CX electron microscope at 80 kV.

Table I. Linkage Analysis of the GelA1000 Mutation in Independently Derived Haploid Segregants of Two Heterozygous Diploids, DG128 and DG133

Linkage group*	Haploid parents	Genotype	DG128 (n = 100)		DG133 (n = 50)	
			gelA1000	+	gelA1000	+
I	HG1264	+	43	0	34	0
	HU1628	cycA1	0	57	0	16
II	HG1264	+	11	21		
	HG1628	acrA1823	32	36		
III	HG1264	+	21	21		
	HU1628	bsgA5	22	36		
IV	HG1264	+	20	38		
	HU1628	whiC351	23	19		
VI	HG1264	+	0	0	5	11
	HU1628	manA2	43	57	22	12
VII	HG1264	+	10	15		
	HU1628	tsgK21	33	42		

* Linkage group V remains to be established and was not marked in this work. DG128 and DG133 are diploid strains that were independently obtained from mutant HG1264 and the tester strain HU1628. Segregants of DG128 were tested for the absence or presence of linkage group markers from the tester strain (genotype column) and for the absence (gelA1000) or presence (+) of the 120-kD gelation factor. Segregants of DG133 were tested only for linkage group I and VI markers.

Results

Isolation of a Mutant Defective in the 120-kD Gelation Factor, and Assignment of the Defect to Linkage Group I

D. discoideum cells mutagenized with MNNG were cloned and blots of the colonies screened in parallel with mAbs 250 and 471, two antibodies that specifically react with the 120-kD gelation factor. Among 16,000 clones scored, a single unlabeled one was detected. This mutant clone was stable; no revertants were observed after growth for more than 200 generations on agar plates. The mutant strain was designated as HG1264 and the defect caused by the mutation as *gelA1000*.

Heterozygous diploids were produced by combining HG1264 with the tester strain HU1628 in which all the six established linkage groups of *D. discoideum* were marked. Colonies of the diploids DG128 and DG133 were labeled by mAb 250 or 471, indicating that the *gelA1000* defect is recessive. After haploidization of the diploids, 150 recombinant cell lines were analyzed. The *gelA1000* defect was found to recombine with any of the linkage group markers except of cycloheximide resistance, the marker of linkage group I (Table I). We conclude that the defect in the 120-kD gelation factor is located on this linkage group, the same as previously found for a defect in α-actinin (Wallraff et al., 1986).

On agar plate cultures, mutant HG1264 could not be distinguished from wild type by morphological criteria. Mutant cells aggregated with streams, formed slugs, and finally fruiting bodies with well-defined stalks and spore heads. The normal morphology may be an intrinsic property of cells carrying the *gelA1000* defect, or it may be due to coselection of a suppressor mutation that stabilizes cell functions against the consequences of a defect in the 120-kD protein. All recombinants listed in Table I showed normal morphology, which excludes a suppressor mutation separable from the *gelA1000* defect by recombination of entire chromosomes. The only possibility remaining is a second mutation on linkage group I, where it could not be separated by parasexual genetics from the *gelA1000* defect. This is an extremely unlikely possibility. Moreover, transformants in which the 120-kD protein was eliminated by gene disruption (Noegel et al., 1989a) resembled HG1264 in exhibiting no gross defects in cell behavior and development.

The 120-kD Gelation Factor Consists of Antiparallel Subunits Containing Repetitive Epitopes

In order to clarify whether only a specific antibody-binding site or the entire 120 kD polypeptide is missing in mutant HG1264, eight antibodies were used that specifically reacted with the 120-kD gelation factor after SDS-PAGE. First, the
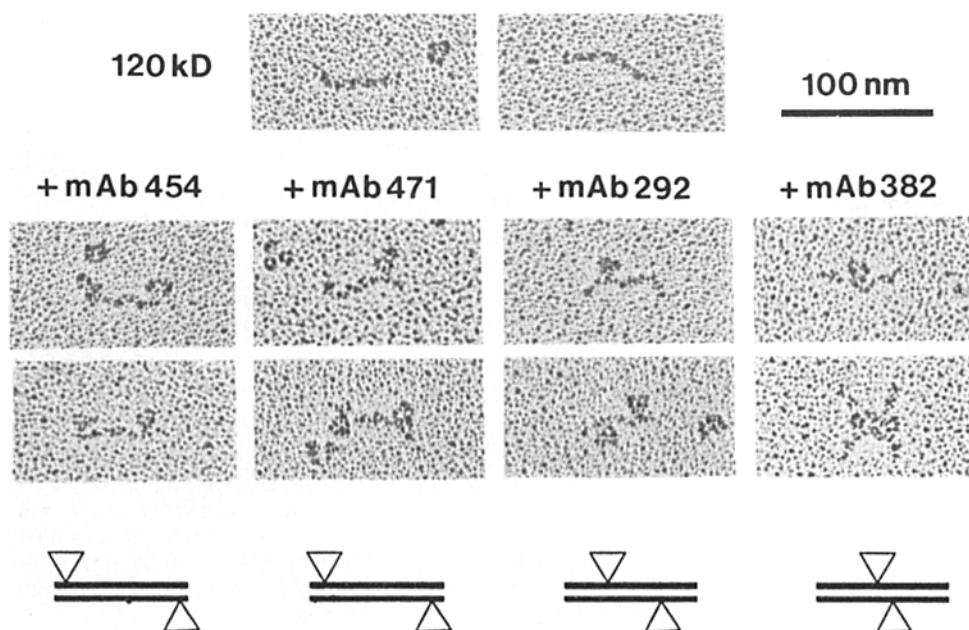
Figure 1. Rotary-shadowed molecules of the gelation factor and their complexes with mAbs 454, 471, 292, and 382. The mAbs bound to the ends, at one third of the length, or in the middle of the rod-shaped molecule. The drawings underneath the micrographs are schematic representations of the different complexes.
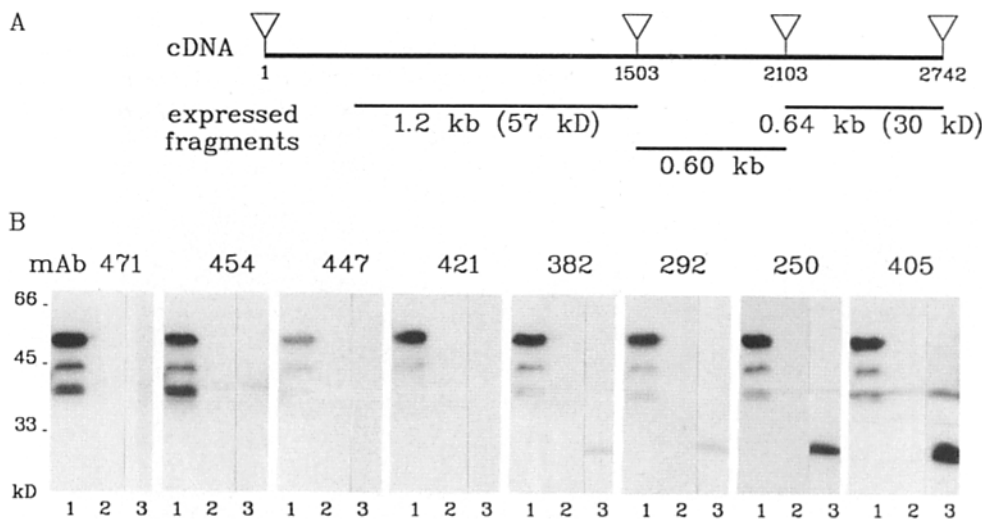
Figure 2. Fragments of the 120-kD gelation factor obtained by expression of cDNA fragments in *E. coli* (*A*) and their labeling with different antibodies (*B*). cDNA inserts were isolated from recombined λgt11-phages after digestion with Eco RI, and cloned into pIMS vectors. The plasmids were then used for the transformation of *E. coli*. Colonies were screened for expression of gelation factor fragments by in situ immunoblotting using a mixture of all mAbs against gelation factor. Total bacterial protein of clones expressing fragments of 1.2, 0.60, or 0.64 kb (lane *1*, *2*, and *3* of each panel, respectively) were separated by SDS-PAGE in 12% gels transferred to nitrocellulose and labeled with the antibodies indicated.

epitopes recognized by these antibodies were mapped on the gelation factor protein from wild-type cells using three different methods. Four of the antibodies formed complexes with the rod-shaped native molecule: mAb 454 and 471 bound at or near to the ends of the rod, mAb 292 bound at about one-third of its length, and mAb 382 in the middle (Fig. 1). In complexes consisting of two molecules of mAb 454, 471, or 292 per rod, one IgG molecule was located near to one end and the second near to the other end of the rod. These data indicate that the filamentous subunits of the gelation factor are connected to each other in antiparallel orientation, as indicated on the bottom of Fig. 1 and shown before for α-actinin (Wallraff et al., 1986).

After limited tryptic digestion of the 120-kD factor and SDS-PAGE of the fragments, three different labeling patterns were observed. One pattern, mostly of large polypeptides, was observed with mAbs 250, 292, 454, and 471, the second pattern, of large and small polypeptides, with mAbs 382 and 405, and the third, mostly of small polypeptides, with mAbs 421 and 447 (data not shown). The distinct labeling pattern obtained with the latter antibodies indicates that at least one epitope in addition to the three epitopes distinguished by EM was recognized.

The amino acid sequence of the 120-kD gelation factor shows six slightly different repeats along the length of the polypeptide from the NH₂- to the COOH-terminal end
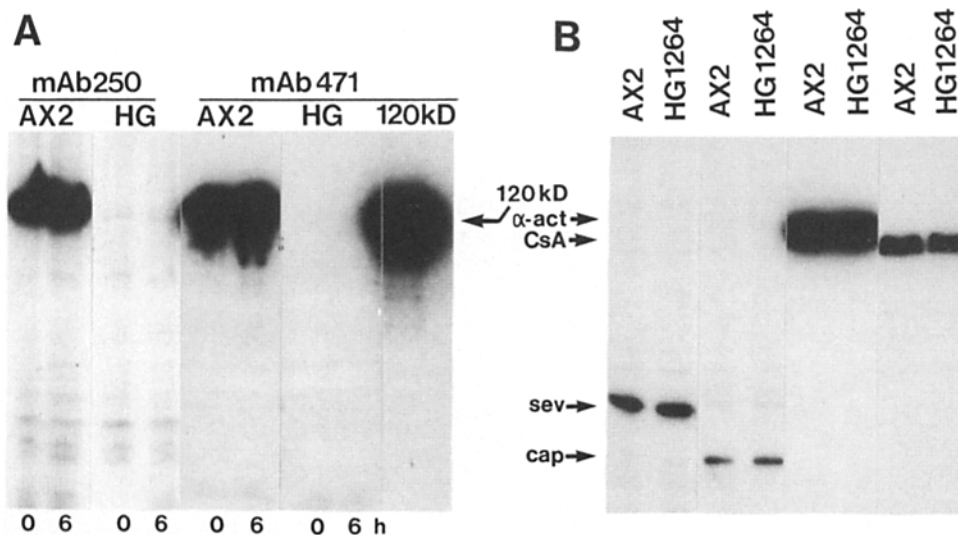
Figure 3. Autoradiograms of blots of total cellular proteins of wild-type (*AX2*) and mutant HG1264 (*HG*). (*A*) Proteins of 5 × 10⁵ cells per slot were separated by SDS-PAGE in 7.5% gels, blotted, and labeled with mAbs 250 and 471. 2 μg of purified 120-kD gelation factor was loaded onto the last slot. Numbers on the bottom indicate h of starvation at which cells were harvested. No 120-kD protein could be detected in the mutant cells. The same result was obtained with the six other mAbs and with polyclonal antibodies. (*B*) AX2 and HG1264 cells were harvested at 6 h of starvation and total cellular proteins were separated on 10% SDS-polyacrylamide gels, blotted, and labeled with mAb 65 for severin (lanes *1* and *2*), with mAb 442 for the 34-kD subunit of the actin capping protein (lanes *3* and *4*), with mAb 19 for α-actinin (lanes *5* and *6*), and with mAb 294 for the contact site A glycoprotein (lanes *7* and *8*). Bands of all proteins were present with normal intensities in mutant as well as wild-type cells.
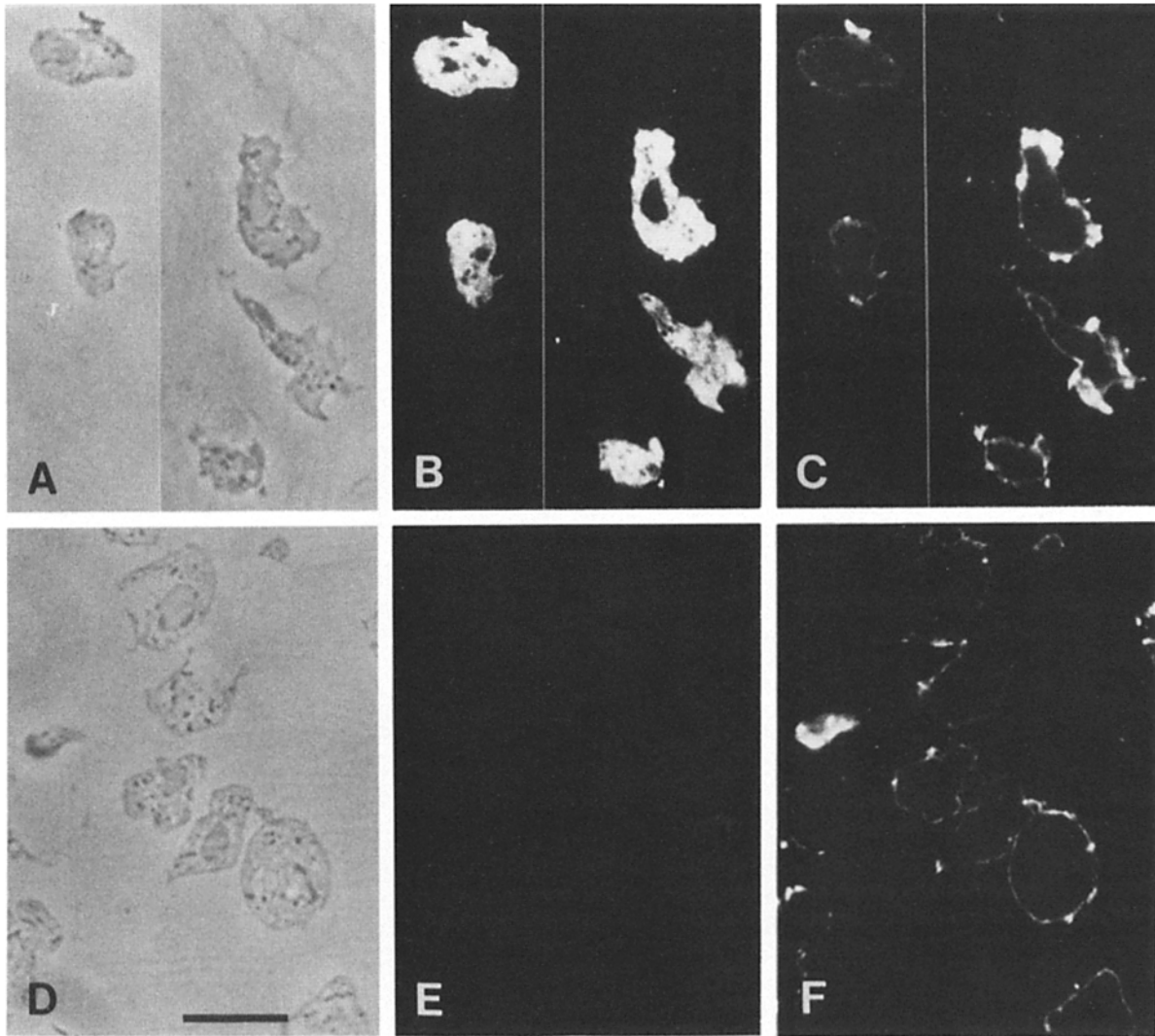
Figure 4. Semithin cryosections of wild-type (*A–C*) and mutant HG1264 (*D–F*) cells fixed at 6 h of starvation. Phase-contrast images (*A* and *D*), immunofluorescent labeling with mAb 454 followed by fluorescein-conjugated second antibody (*B* and *E*), and with rhodamine-conjugated phalloidin (*C* and *F*) are shown. In wild-type cells intense fluorescence is seen in the cytoplasm, whereas nuclei and cell organelles are unlabeled. Mutant sections show no fluorescence. Actin is present as a thin cortical layer closely associated with the plasma membrane in wild-type and in mutant cells (*C* and *F*). Specimens were prepared, labeled, and photographed under exactly the same conditions for wild-type and mutant cells. Bar, 10 μm.

(Noegel et al., 1989*b*). Labeling of fragments of the gelation factor cloned into the pIMS expression vector showed that all antibodies bound to the NH$_2$-terminal half of the subunits, but mAbs 250 and 405 bound also strongly, and mAbs 382 and 292 weakly, to a COOH-terminal fragment (Fig. 2). This labeling pattern suggests that the antibodies bind to subsets of the repeats in the NH$_2$-terminal and COOH-terminal region with different affinities.

The 120-kD Gelation Factor Is Not Detected in Mutant HG1264 by Any of the Antibodies

All the eight antibodies used for the mapping of epitopes were used to label blots of AX2 wild-type and HG1264 mutant proteins after separation by SDS-PAGE. In the wild-type, the 120-kD polypeptide of the gelation factor was recognized by all antibodies in both growth-phase and aggre-

gation-competent cells, whereas no polypeptide of the mutant was labeled by these antibodies, as shown for two of them in Fig. 3 *A*. Since not less than four different epitopes of the gelation factor are recognized by the set of antibodies used, which are distributed along the NH$_2$-terminal half of the molecule (Figs. 1 and 2), and since some antibodies reacted in addition with COOH-terminal fragments of the protein, not more than a minor portion of the 120-kD polypeptide that might be present in the mutant cells. This portion would hardly be able to assemble into antiparallel filaments and would lack the actin-binding site located in the NH$_2$-terminal region. Absence of the entire molecule from mutant cells is also indicated by the lack of a band labeled with affinity-purified polyclonal rabbit antibodies raised against purified 120-kD gelation factor. No label was found with these antibodies after SDS-PAGE and blotting of total proteins of HG1264 cells that were harvested at 0 or 6 h of star-
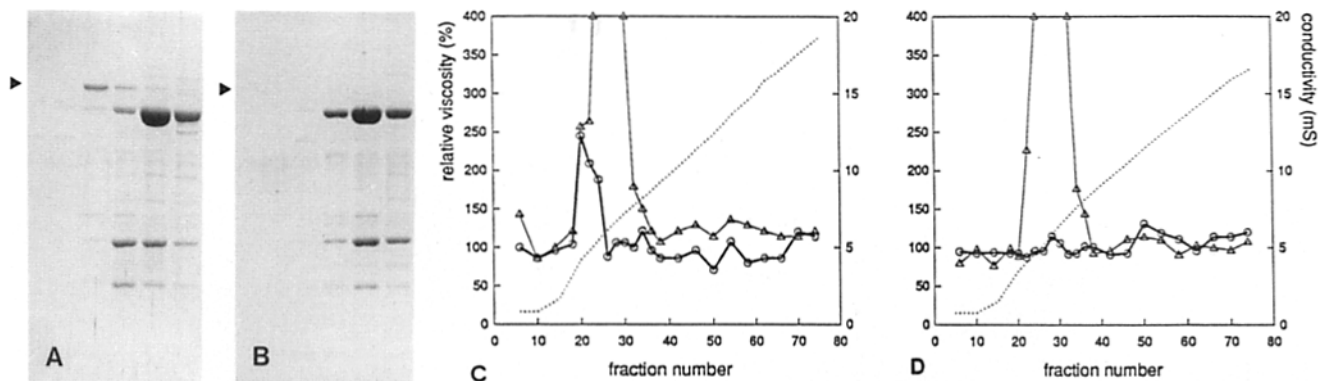
Figure 5. Coomassie blue staining and measurement of cross-linking activity of fractionated wild-type (*A* and *C*) and mutant (*B* and *D*) proteins. Cellular proteins were separated by DE-cellulose chromatography followed by hydroxylapatite chromatography of the fractions which in the wild-type contained viscosity increasing activity. Aliquots of fractions from the hydroxylapatite column were either applied to 10% SDS-polyacrylamide gels and stained with Coomassie blue (*A* and *B*), or tested for crosslinking activity by low shear viscometry in the absence (*triangles*) or in the presence of 0.2 mM Ca^{2+} (*circles*) (*C* and *D*). The 120-kD gelation factor was found in wild-type cells in fractions eluting at 4.2–6.0 mS (*A, arrow*), but no corresponding Coomassie blue–stained band was detected in the mutant (*B, arrow*). In the presence of Ca^{2+}, cross-linking activity was detected in wild-type (*C*) but not in mutant fractions (*D*). In the absence of Ca^{2+}, cross-linking activity was only found in fractions containing α-actinin in both mutant and wild-type cells. All viscosity measurements were done with 0.4 mg/ml of rabbit skeletal muscle actin at least in triplicates.

vation. In blots of wild-type proteins the 120-kD band of the gelation factor was clearly labeled.

Other proteins of the cytoskeleton were normally expressed in the mutant. Thus, the 40-kD band of severin, the 34-kD subunit band of the 32/34-kD capping protein, and the 95-kD band of α-actinin were labeled with normal intensity (Fig. 3 *B*). Expression of the contact site A glycoprotein, which acts as a marker for the cAMP-stimulated development from the growth phase to the aggregation-competent stage, indicates normal regulation of gene expression during development of the mutant.

Immunofluorescence labeling of cryosections confirmed and extended the results obtained on blots. In sections of fixed mutant cells incubated with mAb 454 or mAb 382 followed by fluorescein-conjugated goat anti–mouse IgG, no immunoreactivity due to the 120-kD gelation factor was detected. Both antibodies gave brilliant fluorescence with wild-type cells. In these sections the entire cytoplasm was labeled with the nuclei and smaller organelles remaining unlabeled (Fig. 4 *B*). Labeling with phalloidin-rhodamine revealed no difference in the distribution of F-actin between wild-type

and mutant cells: in both strains the F-actin was concentrated in patches around the periphery of the cells (Fig. 4, *C* and *F*).

No In Vitro Activity of the 120-kD Gelation Factor Is Detected in Cellular Fractions of the Mutant

After separation of wild-type proteins on DEAE-cellulose and hydroxylapatite, the F-actin cross-linking (viscosity increasing) activity of the 120-kD factor and of α-actinin appeared in overlapping peaks (Condeelis et al., 1982), separated from the viscosity-decreasing effects of severin and the 32/34-kD capping protein. The activity of the 120-kD gelation factor can be distinguished from that of α-actinin by its Ca^{2+} insensitivity (Condeelis et al., 1984). The Ca^{2+}-insensitive activity coincided with the presence of the 120-kD band of the gelation factor in Coomassie blue–stained gels (Fig. 5, *A* and *C*), which was labeled in corresponding blots with mAb 471 (not shown). In the corresponding mutant fractions, neither the Ca^{2+}-insensitive viscosity-increasing activity (Fig. 5 *D*), nor the Coomassie blue–stained or antibody-labeled band was detected (Fig. 5 *B*). The Ca^{2+}-sensitive F-actin cross-linking activity of α-actinin and its 95-kD polypeptide band were found in the same fractions irrespective of whether mutant or wild-type homogenates were used. These results confirm that it is specifically the 120-kD cross-linking protein that is missing in the mutant.

An Altered Transcript Is Recognized in the Mutant by cDNA Probes Encoding the 120-kD Protein

In Northern blots a single transcript of 3.0 kb encoding the 120-kD protein was present in all stages of wild-type development. In mutant HG1264, a larger transcript was detected in all stages (Fig. 6). The band of this 3.2-kb transcript was slightly less intense than that of the wild-type. In addition, a faintly labeled transcript was seen in blots of mutant RNA, of a size similar to the wild-type one.

In Southern blots of genomic DNA digested with various restriction enzymes and probed with the complete cDNA, no difference in fragment lengths between the wild-type and
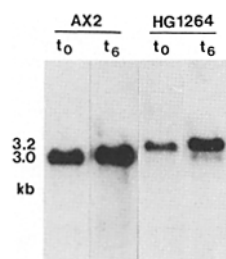
Figure 6. Northern blot analysis of wild-type and mutant RNA. Cells were grown on SM agar with *Klebsiella aerogenes*, harvested, and transferred to Millipore filters for development (Newell et al., 1969). Total RNA from cells that were harvested at 0 or 6 h of development was separated on a 1.2% agarose gel containing 6% formaldehyde, blotted onto nitrocellulose and hybridized with a nick-translated 1.5-kb Eco RI cDNA-fragment of the 5' region of the gelation factor gene. RNA sizes were determined relative to ribosomal RNA. Cells started to aggregate between 6 and 9 h of starvation, formed tipped aggregates after 15 h, and reached the late culmination and fruiting body stages after about 21 h.
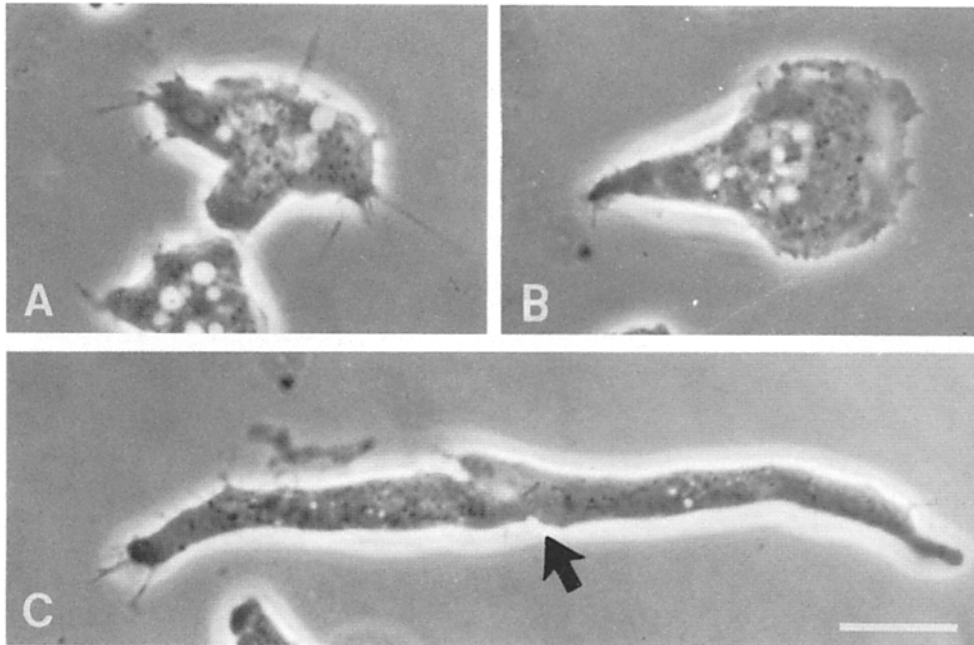
Figure 7. Phase contrast micrographs of cells that were harvested from axenic culture during exponential growth, washed and transferred onto a glass surface either immediately (*A* and *B*), or after 6 h of starvation (*C*). Cell shape is variable and pseudopods are formed in mutant cells similar as in the wild-type. Aggregating cells of the mutant are elongated and form typical cell–cell contacts (*C, arrow*). Bar, 10 μm.

mutant gelation factor gene was detected, which excludes a major deletion or rearrangement of the mutant gene.

Growth of the Mutant

Normally *D. discoideum* cells phagocytose bacteria, laboratory strains are also growing in liquid medium. Since actin and associated proteins might play a role in endocytosis, generation times were determined for wild-type and mutant HG1264 cells growing on *E. coli* or in nutrient medium. No significant change was found in the mutant. The generation time at 23°C in liquid medium was 7.1 h for both strains. The conclusion is that under the optimal culture conditions used neither phagocytosis nor pinocytosis became rate limiting for growth in the absence of the 120-kD gelation factor. In order to test whether the absence of this factor makes the cells sensitive to temperature changes, axenic growth at 23°C was interrupted by daily periods of 8 h at ~4°C. Again no difference in cell numbers of wild-type and mutant was found within 3 d (data not shown).

Membrane Patching and Capping Takes Place in the Mutant

Capping of cell-surface components in response to their cross-linkage by antibodies or other agents is inhibited by cytochalasin D, indicating that the microfilament system is involved in this response (Schreiner and Unanue, 1976). Participation of the 120-kD factor in these processes was suggested by its accumulation in the dense actin network underlying patches (Carboni and Condeelis, 1985). We have used the contact site A cell adhesion molecule, a membrane protein of aggregating cells, as an indicator of normal patching and capping in the absence of this factor. Cells were incubated on ice with mAb 71 directed against the contact site A glycoprotein, and with fluorescein-conjugated polyclonal goat anti–mouse IgG antibodies. In wild-type and mutant HG1264 cells the protein assembled in patches, and accumulated within 15 min in caps after transfer of the cells to 21°C.

Normal Cell Shape and Cortical Actin Filament Assembly in the Mutant

Since indirect evidence has been provided for a role of the 120-kD factor in pseudopod formation (Dharmawardhane et al., 1989) it was surprising that light microscopy did not reveal any dramatic differences between wild-type and mutant HG1264 in cell shape. Growth-phase cells of the mutant showed lamellipods and filopods (Fig. 7, *A* and *B*) like those of the wild-type. Shape changes and localized cell adhesion of aggregating cells were also not prevented by the mutation: aggregation-competent cells became typically elongated and adhered to each other at their ends (Fig. 7 *C*).

In wild-type cells the actin filaments are organized in a network that transverses the entire cytoplasmic space and forms a dense layer in the cell cortex which is broader in lamellipods than in other portions of the cell periphery (Condeelis, 1981). Microfilament bundles extend from this network to build the core of filopods (Claviez et al., 1986). To examine whether the 120-kD gelation factor is required for cortical meshwork or bundle formation, cytoskeletons were prepared from mutant HG1264 and the microfilaments visualized by negative staining. In comparing specimens from wild-type and mutant cells we were unable to distinguish between them. The meshwork of microfilaments in the cortex of a mutant cell and bundles extending from the periphery are shown in Fig. 8.

Cell Movement and Chemotaxis Are Not Substantially Altered in the Mutant

When aggregation-competent *D. discoideum* cells are exposed to a gradient of cAMP they can orient in two different ways. They either turn the front established before stimulation into the direction of the gradient, or this front is paralyzed and a new one is created at the side of the cell that is exposed to the highest cAMP concentration (Segall and Gerisch, 1989). Mutant HG1264 extended normal pseudo-
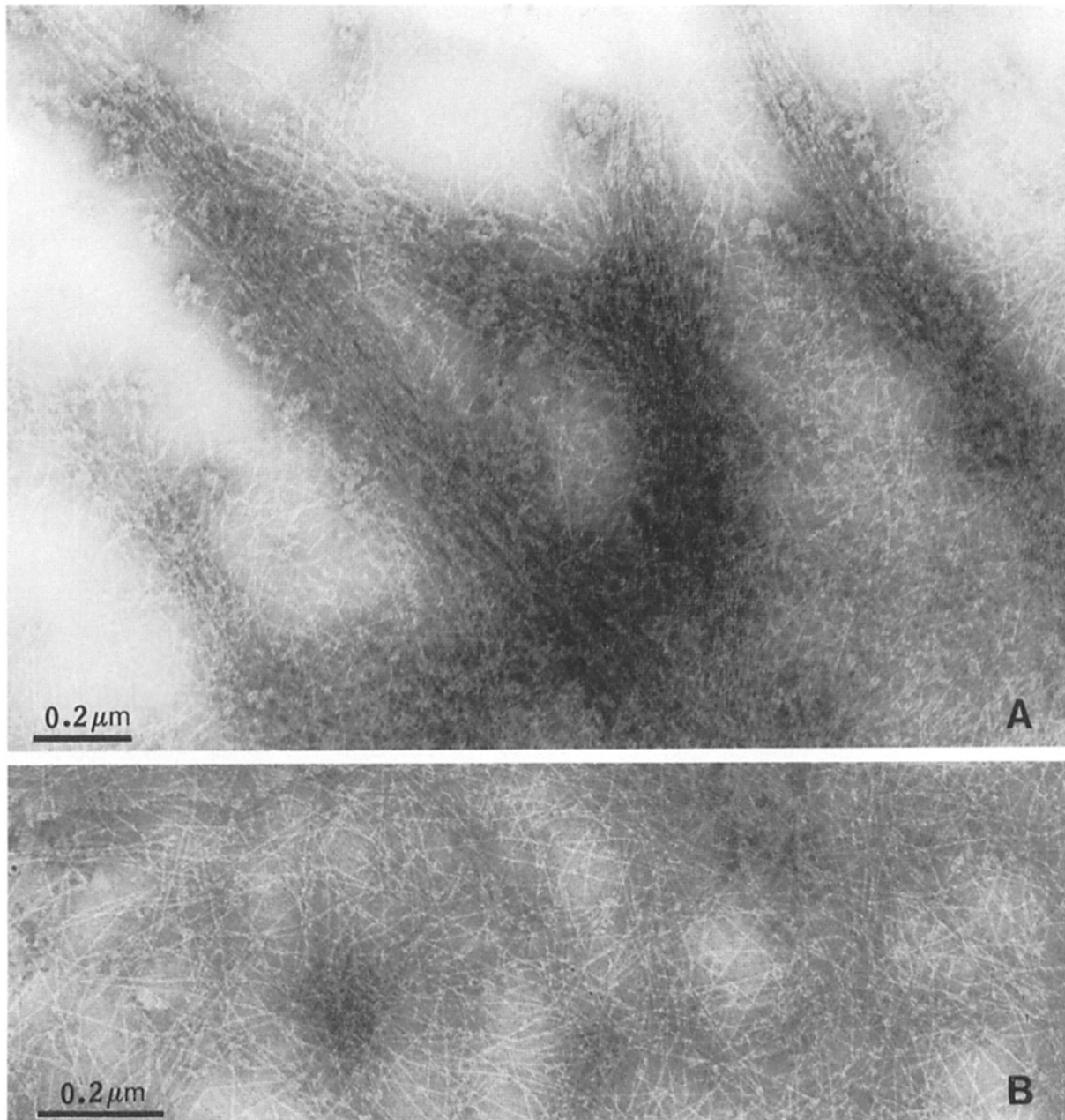
Figure 8. Negatively stained cytoskeleton preparation of a HG1264 cell. The cell was extracted and fixed at 1 h of starvation with 1% Triton X-100 and 0.05% glutaraldehyde in a cytoskeleton stabilizing buffer. Microfilament bundles (*A*) and networks (*B*) in the cortical region are shown.

pods into the direction of a cAMP gradient, and showed the full repertoire of reorientation responses (Fig. 9).

Cell motility and chemotactic orientation in a stable linear gradient of cAMP were quantitatively analyzed using a computer-controlled image-processing system. The orientation of mutant cells was at least as precise as that of wild-type cells, and their speed and turning rate in the presence or absence of a cAMP gradient were also within the normal range (Table II).

High Increase in Actin Content of the Cytoskeleton in cAMP-stimulated Mutant Cells

When aggregation-competent *D. discoideum* cells are stimu-

lated with a pulse of cAMP and soluble proteins are extracted with Triton X-100 at intervals thereafter, a sharp increase of pelletable actin is found at 5–7 s after stimulation, followed by one or two less pronounced increases (McRobbie and Newell, 1983). Because at least part of these responses may be due to changes in the cross-linkage of actin filaments, we stimulated mutant cells with cAMP and compared their responses with that of wild-type cells treated exactly in parallel. A difference was observed in the first peak of actin increase which was higher in the mutant, but no obvious difference in the actin increases at 30–70 s was found (Fig. 10). This is of interest because maximal association of the 120-kD gelation factor with the cytoskeleton has been
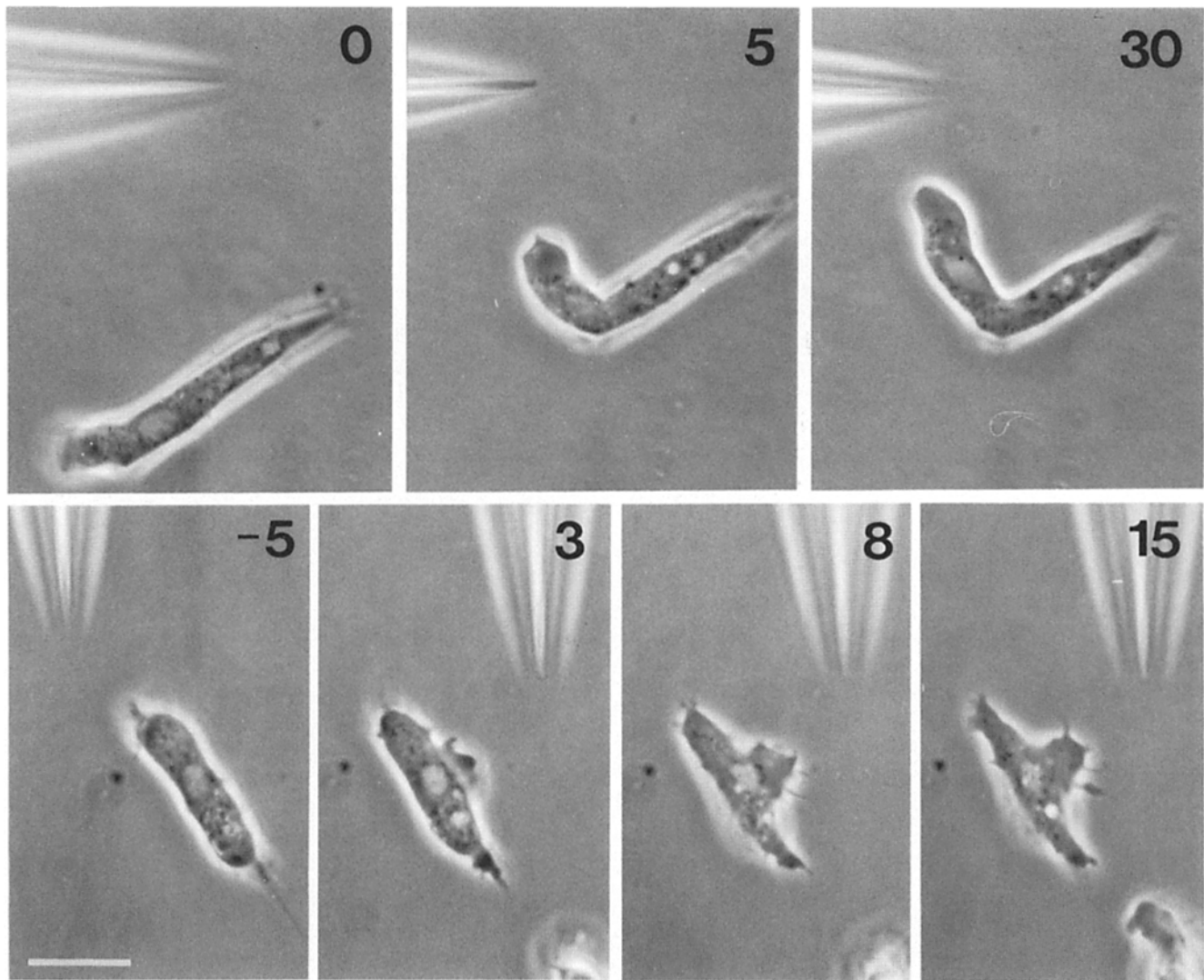
Figure 9. Chemotactic response of HG1264 cells. The cells were washed at 6 h of starvation and transferred onto a glass surface. Cells were stimulated by a micropipette filled with 1 mM cAMP and photographed at intervals. Numbers indicate the time in seconds after insertion of the micropipette. Bar, 10 μm.

Table II. Motility and Chemotactic Orientation of Wild-Type AX2 and Mutant HG1264 Cells

Condition	Strain	Speed	Turning rate	Orientation
		μm/min	*rad²/min*	
Buffer	AX2	7.17 ± 1.94	0.89 ± 0.30	−0.03 ± 0.12
	HG1264	6.28 ± 2.13	0.80 ± 0.05	−0.03 ± 0.09
In a cAMP	AX2	10.40 ± 2.10	0.84 ± 0.16	0.29 ± 0.06
gradient	HG1264	10.00 ± 2.87	0.71 ± 0.14	0.34 ± 0.04

Axenically grown cells were starved for 6 h and deposited on a glass surface coated with BSA, which was then placed into a chemotaxis chamber. Speed of cell movement, orientation, and turning rate was recorded in buffer for 30 min before the gradient was applied. Then measurements were interrupted for 30 min during which the cAMP gradient was established (Fisher et al., 1989). Recording was continued thereafter for another 30 min. The turning rate describes as a rotational diffusion coefficient the rate at which the direction of movement of a cell changes (Segall et al., 1987). Orientation is the fraction of the distance travelled per time-lapse interval that is in the direction of the gradient. Data are averages of nine and five independent experiments for AX2 and HG1264, respectively, ± standard deviation. The number of cells from which data were collected was 20–70 per experiment. The differences between wild-type and mutant data are in no case significant with a 10% level of confidence as calculated in a two-tailed *t* test.

reported to occur at 30–70 s of stimulation (Condeelis et al., 1988; Dharmawardhane et al., 1989). By quantitating in 10 pairs of parallel experiments the mean increase during the first peak, pelletable actin was found to increase by 48% in the wild-type and 69% in the mutant. In all but one pair of experiments this stronger response of mutant cells was found (Table III). The portion of pelletable to total actin in unstimulated cells did not significantly differ between wild-type and mutant.

Discussion

In previous reports *D. discoideum* mutants with defects in α-actinin (Wallraff et al., 1986; Schleicher et al., 1988*a*), myosin II (De Lozanne and Spudich, 1987; Knecht and Loomis, 1987), or severin (André et al., 1989) have been described. Regions of all three proteins are conserved during evolution, suggesting important functions of these proteins not only in amebae but also in vertebrates (Schleicher et al., 1988*b*). Myosin II-deficient cells are severely altered in cyto-
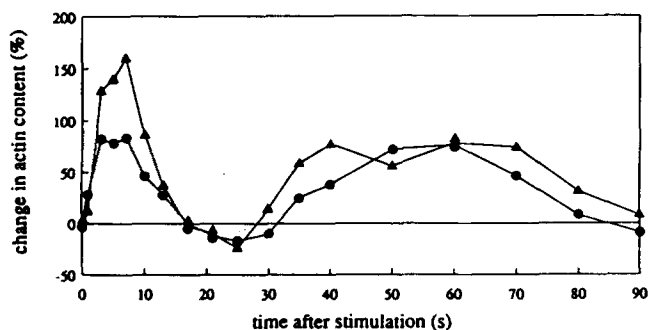
Figure 10. Time course of the accumulation of actin in the Triton-insoluble cytoskeleton of aggregation-competent AX2 (*circles*) and HG1264 (*triangles*) amebae at 8 h of development in response to a pulse of 10^{-7} M cAMP. Cytoskeletons were subjected to SDS-PAGE. The Triton X-100 insoluble pellets of 2.5×10^5 cells were loaded per lane and Coomassie blue–stained bands were measured in an electrophoresis scanner.

kinesis, but still able to move, although slower than wild-type cells (De Lozanne and Spudich, 1987; Knecht and Loomis, 1987). Chemotactic orientation of the myosin II-deficient cells is, on the average, less precise (Wessels et al., 1988; Peters et al., 1988). Quite in contrast, no substantial changes in growth, motility, or development have been found in the α-actinin- and severin-deficient mutants, which implies that the missing proteins are not essential for these processes.

Here mutant HG1264 of *D. discoideum* has been characterized, which lacks the 120-kD gelation factor, the second actin-cross-linking protein next to α-actinin in abundance (Condeelis et al., 1981). This protein has come into focus since a role has been attributed to it in the process of pseudopod formation. Condeelis et al. (1988) have shown that the

Table III. Actin Content of the Triton X-100-insoluble Cytoskeleton of Resting Wild-type AX2 and Mutant HG1264 Cells, and its Increase in Response to a Pulse of 10^{-7} M cAMP

AX2		HG1264	
Ratio of pelleted to total actin in resting cells	Increase after stimulation (% of resting level)	Ratio of pelleted to total actin in resting cells	Increase after stimulation (% of resting level)
20	51	26	73
24	–	23	70
26	37	29	46
24	50	28	48
28	19	24	55
–	43	–	51
–	61	–	73
–	61	–	73
–	83	–	160
–	25	–	37
–	46	–	68

The Triton X-100 insoluble cytoskeletal fractions were prepared after 6 or 8 h of starvation before and at 7 s after stimulation by a pulse of 10^{-7} M cAMP. The cytoskeletal proteins were subjected to SDS-polyacrylamide electrophoresis in 10% minislab gels. The Triton-insoluble pellets of 2.5×10^5 cells were loaded per lane, and Coomassie blue–stained actin bands were measured in a gel scanner. Under the Wilcoxon matched-pairs signed-rank test, the difference in the increase after stimulation between wild-type and mutant is statistically significant with a chance probability ≤0.005.

120-kD protein is colocated with actin in pseudopods, particularly in newly formed ones after stimulation of a cell with cAMP. Participation of the 120-kD factor in cross-linking actin filaments in the cell cortex has also been suggested by electron microscopic studies showing that the cortical meshwork of *D. discoideum* cells resembles the network which is formed in vitro by a mixture of purified actin with the 120-kD gelation factor (Wolosewick and Condeelis, 1986).

In mutant HG1264 the 120-kD gelation factor has not been detectable on immunoblots with polyclonal antibodies, and also not with mAbs that recognize at least four different epitopes distributed over the NH_2-terminal, middle, and COOH-terminal region of the polypeptide. Similarly, by incubation of cryosections with the antibodies no immunoreactivity has been found in the mutant cells. Undetectability of Ca^{2+}-independent viscosity increasing activity that could be due to the gelation factor excludes the presence of a severely altered but still active protein. The mutant produces one transcript that is larger than the normal mRNA encoding the 120-kD protein, and a second, very faint transcript of a size similar to that of wild-type mRNA. In analogy to the α-actinin-deficient mutant HG1130, where a splice site is altered (Schleicher et al., 1988a; Witke and Noegel, 1990), the transcript with an increased size might result from a splicing defect in HG1264. Unspliced transcripts could be translated into truncated polypeptides that are incorrectly folded and therefore rapidly degraded. Absence of any detectable quantities of the 120-kD polypeptide suggests that also the mutant transcript of almost normal size does not give rise to a correct translation product.

In HG1264 mutant cells extracted with Triton X-100, we have found a cortical meshwork and bundles of microfilaments similar to wild-type structures. Cryosections of fixed cells show that absence of the gelation factor does not alter the distribution of F-actin, which in mutant and wild-type forms a layer close to the cell membrane. These results show that the presence of a cortical network within the cells does not depend on the 120-kD gelation factor. The gelation factor has been detected by immunolabeling of cryosections throughout the cytoplasm of wild-type cells, indicating that under normal conditions only a small portion, if any, of the 120-kD protein is associated with the cortical actin filaments. No gross alterations of growth on bacteria or in axenic culture have been found in the mutant. From this we conclude that phagocytosis or pinocytosis of cells is not severely affected by the mutation. The cell shape is not substantially altered in the mutant; its cells form normal pseudopods within a few seconds after stimulation by cAMP through a micropipette (Fig. 9). Speed of movement and turning rate are also not significantly changed in the mutant cells, and if there is any change in chemotactic orientation, its precision is increased rather than reduced (Table II). The same is true for the fast rise in actin content of the cytoskeleton in cAMP-stimulated cells: the increase is significantly stronger in the mutant than in wild type (Table III). From these results we conclude that the 120-kD gelation factor is not important for the cell functions investigated but plays a modifying role in assembly of the actin cytoskeleton. We consider a protein important if it cannot be replaced by another protein, or can be replaced only with a substantial loss in strength or precision of a particular cell function as discussed by Gerisch et al. (1989).

The genetic studies presented in this paper provide a basis for further investigations on the functional organization of the cytoskeletal system of motile nonmuscle cells. The principal way to analyze this system is to successively eliminate several if not all proteins with similar in vitro functions to overcome the problem of redundancy. A second way is to expose the mutant to a series of unfavorable conditions that are sublethal for the wild-type, to find out whether certain proteins, not required under optimal conditions, protect the cells against damage caused by stress. A third way is to evaluate small differences in the selection value by subjecting the mutant to long-term competition with wild-type under quasi-natural conditions. A small reduction in growth rate has been found for the α-actinin-deficient mutant of *D. discoideum* (Schleicher et al., 1988a), which might be sufficient to extinguish under long-term selection a cell population in which the protein is missing.

In the light of the obvious need of further analyses we wish to emphasize the main goal of the present study. Our attempt is to define the minimal set of actin-binding proteins required for motility, for the determination of cell shape and the control of chemotactic orientation. The strategy is to establish by elimination of one protein after another a cell line in which the role of the essential proteins left in the cells can be investigated more conveniently than in the wild-type, where their activities may be obscured by the presence of other proteins with overlapping functions (Bray and Vasiliev, 1989). Isolation of mutants defective in a single, dispensable protein, as they are described in the present paper and in the two previous ones (Wallraff et al., 1986; André et al., 1989), is a first step in simplifying the in vivo situation for experimental purposes. However, our findings are limited to the defined laboratory conditions used. It is therefore not possible to question from these results the importance of a protein under natural conditions. It is also not our intention to exclude subtle effects the loss of an abundant protein will certainly have. All three proteins are characterized by highly conserved domains, which suggests that they have general functions of selective value.

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